The Effects of Mechancial Stimulation and Cytokine Stimulation on Proteoglycan Levels in Human Chondrocytes

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Declaration

(I) This thesis is my own work

(II) The experimental work described was carried out by myself, unless otherwise stated

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Abstract

Osteoarthritis (OA) is characterised by the degeneration of the cartilage matrix leading to cartilage destruction and loss. The underlying bone is therefore no longer protected from the forces associated with joint movement. Chondrocytes are the only cell type found in normal cartilage. These are considered to be terminally differentiated cells that play a role in the synthesis and maintenance of the cartilage matrix. The repeated and abrupt force changes that chondrocytes are subjected to provide stimuli that can modify the cells metabolic function. In addition to mechanical stimuli, chondrocyte function can also be regulated by a variety of cytokines and growth factors. Several proinflammatory cytokines have been shown to mediate their effects through the induction of nitric oxide (NO) production. The aim of this study was to investigate the interactions between cyclic mechanical stimulation, nitric oxide production and the regulation of human chondrocyte proteoglycan levels.

Transformed chondrocyte cell lines and primary OA human articular chondrocytes (HAC) were grown in monolayer culture. Cells were exposed to cyclical mechanical stimulation (MS) using an apparatus that produces strain on the base of the culture dish, causing the deformation of attached cells. Chondrocytes were also incubated with a cytokine cocktail containing IL-1β, TNFα, IL-6 and IFNγ (CYT). Nitric oxide synthase (NOS) mRNA, protein and nitrite levels were measured, as well as aggrecan mRNA levels.

Unexpectedly, the transformed chondrocyte cell lines C20A4 and C28I2 did not express detectable levels of NOS protein or nitrite activity, while increased inducible NOS (iNOS) mRNA following CYT stimulation suggested that the cells were able to sense this stimulus.

Primary OA HAC showed increased production of iNOS mRNA, protein and nitrite following CYT or IL-1β stimulation. The simultaneous application of CYT and MS also showed elevated iNOS mRNA, protein and nitrite levels, although these were significantly lower than following CYT alone. An IL-4 neutralising antibody and a β1 integrin function blocking antibody did not reverse the decreased CYT induced iNOS associated with MS. This suggests that MS decreases CYT induced iNOS through an IL-4 and a β1 integrin independent mechanism.

A novel iNOS inhibitor (AR-C102222) was shown to have little effect on iNOS mRNA and protein levels following CYT stimulation of primary OA HAC. However, a dose dependent decrease in nitrite production was seen.

Aggrecan mRNA levels in primary OA HAC were decreased following stimulation with CYT. The possible role of NO in this decreased aggrecan mRNA level was investigated. MS and a novel iNOS inhibitor both decreased the production of NO, but did not alter the CYT mediated decreased aggrecan mRNA. The NO donor SNAP did not alter aggecan mRNA levels. These results suggest that NO is not involved in regulating aggrecan mRNA levels, although several factors may influence the aggrecan mRNA levels which makes it difficult to confirm this conclusion. The final study using cells from an iNOS knockout mouse suggested that both NO dependent and independent mechanisms exist by which CYT can decrease aggrecan mRNA levels.

These studies demonstrate that MS may have an anti-inflammatory role through decreasing CYT induced iNOS. Further work is required, however, to understand the complex role which NO may play in the regulation of cartilage matrix homeostasis.

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Contents

Abbreviations

ACL	Anterior cruciate ligament transection
ADAM-TS	A disintegrin and metalloproteinase with thrombospondin like domain
AIA	Adjuvant induced arthritis
Arg	Arginine
BMP	Bone morphogenic protein
Ca^{2+}	Calcium
CaM	Calmodulin
CIA	Collagen induced arthritis
COXII	Cyclooxygenase II
C_T	Threshold cycle
CTS	Cyclic tensile strain
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular regulated kinase
FAC	Focal adhesion complex
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FMN	Flavin mononucleotide
GAG	Glycosaminoglycan
GAS	Gamma activated sequence
GSH	Glutathione
HA	Hyaluronic acid
HAC	Human articular chondrocytes
HDAC	Histone deacetylase
hiNOS	Human iNOS
HO-1	Haem oxygenase 1
ICE	Interleukin 1 converting enzyme
IFNγ	Interferon gamma
IGF-1	Insulin-like growth factor 1

IHP	Intermittent hydrostatic pressure
IκB	Inhibitor of κB
IL-1β	Interleukin 1β
IL-1ra	Interleukin 1 receptor antagonist
IL-1RacP	Interleukin 1 receptor accessory protein
IL-1RI	Interleukin 1 receptor type I
IL-1RII	Interleukin 1 receptor type II
ILK	Integrin linked kinase
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IP_3	Inositol triphosphate
IRAK	Intracellular receptor for activated kinase
IRF-1	Interferon regulatory factor 1
JAK	Janus activated kinase
JNK	c-Jun NH_2 terminal kinase
KO	Knockout
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MEKK1	MAPK and ERK kinase
MMP	Matrix metalloproteinase
MS	Cyclic mechanical stimulation at 0.33Hz and 30,000μstrain
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazonium bromide]
NADPH	Nicotinamide adenosine dinucleotide phosphate
NAP110	NOS associated protein 110
NF IL-6	Nuclear factor IL-6
NFκB	Nuclear factor κB
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NRE	Negative regulatory element
NRF	NFκB repressing factor
O_2^-	Superoxide radical
OA	Osteoarthritis

$ONOO^-$	Peroxynitrite
PBS	Phosphate buffered saline
PG	Proteoglycan
PGE_2	Prostaglandin E_2
PIAS	Protein inhibitors of STAT
PKB	Protein kinase B
PKC	Protein kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
RA	Rheumatoid arthritis
RACK-1	Receptor for activated c-kinase 1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAC	Stress activated ion channels
SAPK	Stress activated protein kinase
SCW	Streptococcal cell wall
SEK	SAPK/ERK kinase
SEM	Standard error of the mean
SNAP	S-nitroso-N-acetylpenicillamine
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SOCS	Suppressors of cytokine signalling
STAT	Signal transducer and activator of transcription
TBS-T	Tris buffered saline plus tween-20
TGFβ	Transforming growth factor β
TIMP	Tissue inhibitor of MMP
TIR	Toll/IL-1 receptor domain
TNFα	Tumour necrosis factor α
Tollip	Toll-interacting protein
TRAF	TNF receptor associated factor
UTR	Untranslated region
WT	Wild-type
ZIA	Zymosan induced arthritis

NOS Inhibitor Abbreviations

AG	Aminoguanidine
GW273629	3-[[2-(ethanimidoylamino)ethyl]sulphonyl]-L-alanine sulphone
GW274150	3-[[2-(ethanimidoylamino)ethyl]sulphonyl]-L-alanine sulphide
L-NAME	NG-nitro-L-arginine methylester
L-NIL	L-N(6)-(1-iminoethyl)lysine
L-NIO	NG-L-nitro-L-arginine methyl ester
L-NMA and L-NMMA	N(G)-monomethyl-L-arginine
7-NI	7-nitroindazole
7-NIBr	3-Br-7-nitroindazole
1400W	N-3-aminoethyl-benzyl-acetamidine hydrochloride

Chapter 1 - Introduction

1.1 – Articular Cartilage

1.1.1 - Structure of Articular Cartilage

Articular cartilage is a highly specialised and uniquely designed bio-material that forms the smooth, gliding surface covering the heads of long bones in articular joints. This avascular, aneural and alymphatic tissue provides protection for the underlying bone against shearing and compressive forces. Articular cartilage is composed of water (65-80%), collagen (10-30%), proteoglycans (5-10%) and chondrocytes, the cells responsible for cartilage synthesis and homeostasis (0.4-2%) (Kuettner et al 1991; Poole 1997). The cartilage is normally divided into 4 zones parallel to the surface (**Figure 1.2**) (Collins and McElligott 1960):

(I) Superficial zone (5-10%); adjacent to joint cavity, where collagen fibrils are arranged tangentially to the surface. The cells are discoidal with their long axis parallel to the surface.

(II) Intermediate (transitional) zone (40-45%); the coiled collagen fibrils are randomly oriented in an interlacing meshwork, with the cells spheroidal and evenly spaced.

(III) Deep zone (40-45%); the cells are large, rounded and aligned in vertical columns. The collagen fibres are arranged radially to the surface.

(IV) Calcified zone (5-10%); adjacent to the subchondral bone. There are few cells and the matrix is mineralised with crystals of calcium salts. The border between the deep and calcified zone is visible in histological sections and is called the tide mark.

The matrix of the intermediate and deep zones can be further divided into the pericellular, territorial and interterritorial, each with different structural organisation (Poole et al 1982) (**Figure 1.1**). The pericellular matrix and the chondrocyte form a structure known as the chondron, composed of a fine collagen type II mesh containing the cell. Collagen VI is found only in the pericellular matrix and is thought to link collagen II to the chondrocyte. The different zones of cartilage have altered ECM composition, with increased collagens in superficial areas and increased PGs in deep

areas exposing the cells within each zone to different strains (Langelier et al 2000). The cartilage extracellular matrix (ECM) is composed of fibrillar and non-fibrillar components.

The fibrillar matrix is a network consisting primarily of triple helical type II collagen molecules (Aigner et al 2002b). This is the major structural component of cartilage, forming a highly cross-linked fibrillar network that is found throughout the ECM, providing the strength of the tissue by resisting shear and tensile forces (Kempson et al 1973). The tangential arrangement of these fibres in the superficial layer resists shear stresses during joint movement whereas the radial organisation in deeper layers functions primarily to resist the internal swelling pressure of the matrix (Meachim et al 1974). Collagen types VI, IX, XI, XII and XIV are minor components of the cartilage ECM, however they appear to have important structural and functional properties (Goldring 2000).

The non-fibrillar matrix consists mainly of aggrecan monomers highly sulphated through the covalent attachment of glycosaminoglycan (GAG) sidechains of chondroitin sulphate and keratan sulphate (Doege et al 1991). Aggrecan accounts for approximately 80% of proteoglycans (PG) in articular cartilage (Carney and Muir 1988), forming large macromolecular aggregates via link protein stabilisation of its non-covalent interaction with polymeric hyaluronic acid through the G1 globular domain of the aggrecan core protein (Poole et al 1980). Full-length aggrecan attached to hyaluronan binds the cell surface receptor CD44 (Embry and Knudson 2003). The GAG molecules consist of long chain, unbranched, repeating dimeric polysaccharides containing both carboxyl and sulphate groups to provide a highly negatively charged aggregate. Polymorphism in the chondroitin sulphate domain makes aggrecan a heterogeneous population (Hardingham and Bayliss 1990). The fixed negative charge of the PGs dictate the extracellular ionic composition of cartilage, causing high osmolality that results in fluid being imbibed by cartilage. This swelling is resisted by the collagen fibres, so even at rest there is a hydrostatic pressure of 0.2MPa in human articular cartilage. Under compressive force cartilage is compliant, rapidly recovering its elasticity as water is drawn back into the matrix by the hydrophilic aggrecan aggregates (Carney and Muir 1988). The PGs also act as permeability barriers

preventing molecules such as immunoglobulins from entering the tissue (Urban 1994).

The collagen network therefore provides tensile strength that hinders the expansion of the viscoelastic aggrecan, providing compressive stiffness and the ability to resist deformation and dissipate load (Maroudas 1976; Poole 1997).

There are several other matrix proteins that are not unique to cartilage, but have important roles in cartilage stucture and function. The small leucine-rich proteoglycans biglycan, decorin, fibromodulin and lumican interact with the collagen network, contributing to the fixed charge density and binding growth factors such as transforming growth factor β (TGF-β) (Heinegard and Oldberg 1989). Non-collagenous proteins are also found, with fibronectin promoting attachment to chondrocytes through the RGD amino acid sequence (Burton-Wurster et al 1998).

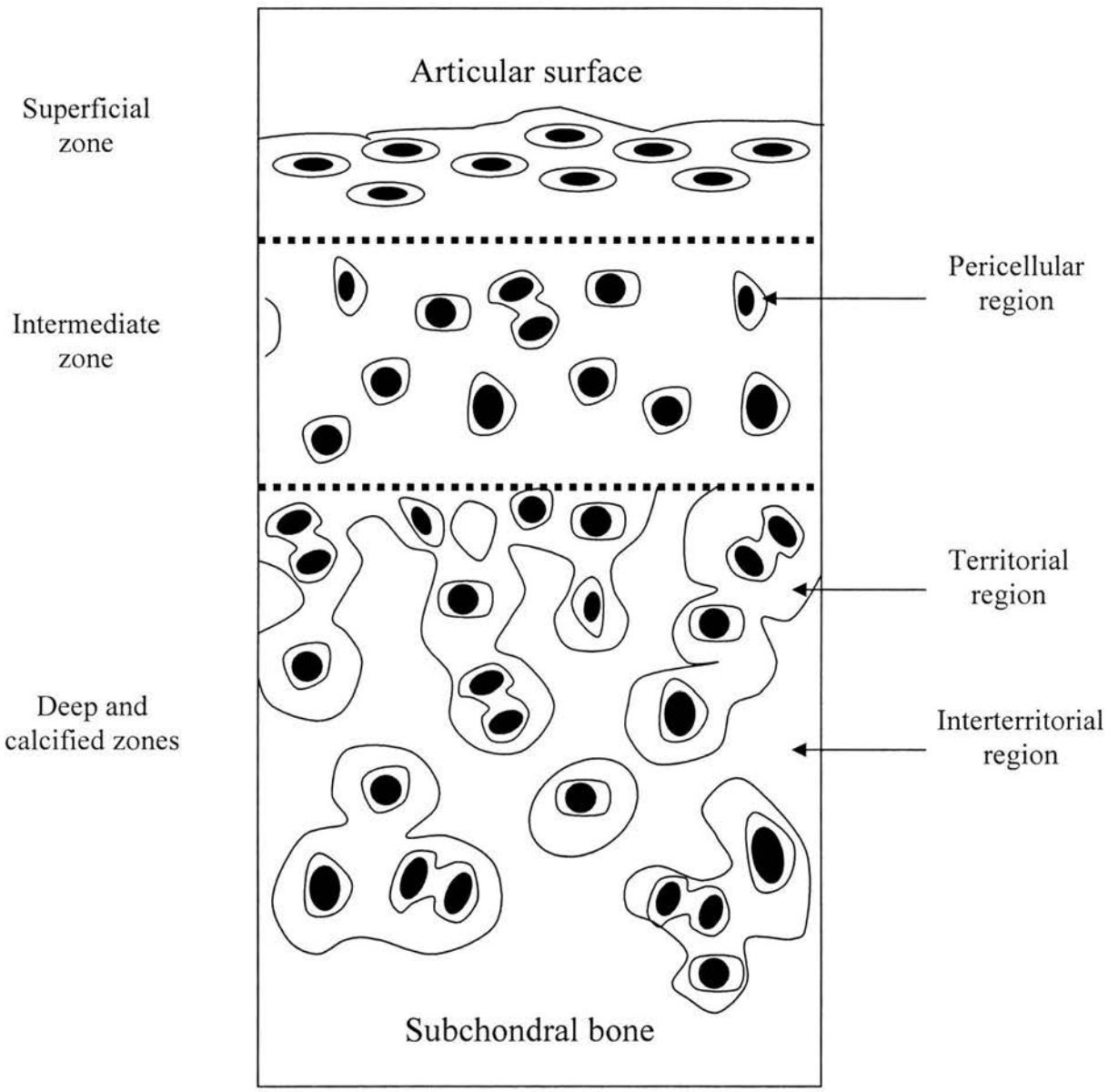


Figure 1.1 – Diagrammatic representation of the zones and regions of articular cartilage

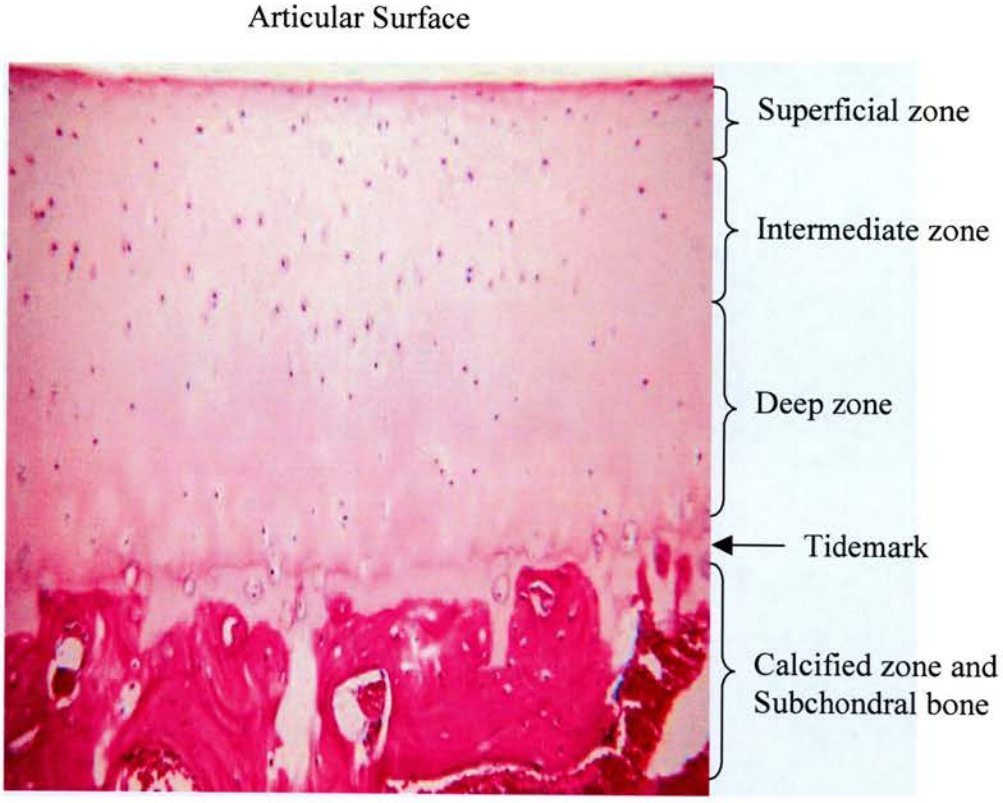


Figure 1.2 – Hematoxylin-eosin stained section of human articular cartilage

1.1.2 - Chondrocytes

Chondrocytes are the only cell type found in normal cartilage. These are considered to be terminally differentiated cells that play a role in the synthesis and maintenance of the cartilage matrix (Goldring 2000). A dynamic balance exists between PG synthesis and degradation allowing chondrocytes to maintain a uniform concentration of PGs within the cartilage matrix (Morales and Hascall 1989).

The matrix metalloproteases (MMPs) are a family of enzymes that degrade cartilage ECM including collagens (Billinghurst et al 1997) and PGs (Lark et al 1997). These MMPs are synthesised as latent proenzymes activated through proteolytic cleavage by molecules such as plasmin and cathepsin B (Mehraban et al 1994). These enzymes are kept under control by the production of molecules termed tissue inhibitors of MMPs (TIMPs) (Attur et al 2000). The production of MMPs is upregulated by proinflammatory cytokines such as interleukin-1β (IL-1β) (Little et al 2002). The inhibition of IL-1β activity through addition of IL-1 receptor antagonist (IL-1ra) (Bresnihan et al 1998) or transfection with the non-functional IL-1 receptor II (IL-1RII) (Attur et al 2000; Attur et al 2002) leads to a decrease in MMP production. ADAM-TS (a disintegrin and metalloproteinase with thrombospondin type) 1, 4 and 5 are enzymes involved in aggrecan cleavage (Tortorella et al 1999).

The variation in PG concentration within cartilage and across the joint indicates that sensitive mechanisms exist to regulate PG turnover which are dependent on the chondrocytes' physicochemical environment (Langelier et al 2000).

The repeated and abrupt force changes that chondrocytes are subjected to provide mechanical stimuli that can modify the cells metabolic function. Attachment of the ECM to the cell surface provides a mechanism through which mechanical signals could be sensed by the cells. The dynamic aggrecan proteoglycan structure assembled on hyaluronic acid (HA) binds to its receptor, CD44, on the cell membrane (Knudson 1993). Several integrin molecules have been identified on the cell surface that bind specific components of the ECM (Knudson and Loeser 2002; Salter et al 1992). An example of the response to strain is the frequency dependent increase in PG synthesis that occurs following compression (Urban 1994).

In addition to mechanical stimuli, chondrocyte function can also be regulated by a variety of cytokines and growth factors. These include the proinflammatory cytokines such as IL-1β and tumour necrosis factor α (TNFα) which act through the generation of other inflammatory mediators resulting in decreased synthesis and increased breakdown of PGs (Goldring 2000). Growth factors including TGFβ and insulin-like growth factor-1 (IGF-1) stimulate chondrocyte differentiation and the synthesis of cartilage matrix components, such as PGs and collagen type II (Aigner et al 2002b).

Chondrocytes are also influenced by other factors in their extracellular environment. The concentration of fixed negative charges on the proteoglycans influences H^+ ion concentrations and so extracellular pH. Chondrocytes obtain their energy primarily via anaerobic glycolysis, with lactic acid diffusing out to the synovial fluid causing pH gradients. The avascular nature of cartilage results in gradients of other molecules including oxygen varying from 6% in superficial to 1% in deep layers (Urban 1994).

1.2 - Osteoarthritis

Radiographic evidence of osteoarthritis (OA) is seen in at least one joint in more than 70% of persons from 55-74 years old and contributes greatly to the cost of health care for ageing populations (Gabriel et al 1997, Lawrence et al 1998). The incidence in women is higher than in men, especially for hand and knee OA, and particularly after 50 years of age (Butler et al 1988). Extrinsic factors such as physical activity and injury influence OA. Intrinsic factors that determine load distribution include alignment, muscle strength and varus-valgus laxity, and can all contribute to OA (Sharma 2001). The risk of developing knee OA is increased due to increased weight (Anderson and Felson 1988) and genetic studies have identified multiple gene variations associated with an increased risk in OA. This suggests there is potentially a genetic component involved in OA progression (Reginato and Olsen 2002).

The macroscopic alteration in the cartilage surface in OA includes fibrillation, erosion and cracking. The most widely used system for grading OA from gross pathology is that proposed by Collins and McElligott (1960). The microscopic structure of cartilage is commonly graded using the system described by Mankin et al (1971). The combined use of both macroscopic and microscopic techniques will allow more competent assessments of joint changes in OA (Ostergaard et al 1997a).

The disease involves the slowly progressive loss of articular cartilage. Cartilage swelling is an early event in OA, probably occurring due to collagen damage that allows PGs to swell further (Maroudas and Venn 1977). Increased swelling dilutes GAGs and cartilage has a reduced load bearing capacity and decreased hydraulic permeability (Armstrong and Mow 1982). Degeneration is first observed at the articular surface in the form of fibrillation. Superficial fibrillation is associated with increased denaturation and loss of type II collagen as collagen fibrils are degraded leading to a loss of tensile properties of the cartilage (Hollander et al 1995). The damage of the matrix is associated with the release of PGs including decorin, biglycan and aggrecan (Kempson et al 1973)). The early damage and loss of matrix molecules is accompanied by increased aggrecan, decorin and biglycan and type II collagen in the mid and deep zones, thought to be due to the increased load on these cells following the damage to the superficial layer (Nelson et al 1998).

The OA lesion is often localised to the weight bearing cartilage or to sites of trauma, suggesting mechanical factors may be the critical signal for OA initiation and progression (Goldring 1999; Urban 1994). Although OA is frequently regarded as a non-inflammatory arthritis considerable data implicate a role for proinflammatory cytokines derived from both the synovium and chondrocytes in cartilage destruction during OA. OA cartilage has been reported to express inducible Nitric oxide synthase (iNOS) following stimulation with IL-1β and TNFα, which results in the release of Nitric oxide (NO) which, as will be discussed later, may contribute to cartilage pathology (Amin et al 1997).

The size of aggrecan molecules and their pattern of sulphation alter with ageing (Roughley 2001), while collagen crosslinking increases with age, making cartilage stiffer and more brittle (Eyre et al 1998). These changes will alter loading conditions for the cells, so are likely to induce early pathologic changes in OA (Aigner et al 2002b).

Collagen damage is seen around the chondrocytes extending into the interterritorial sites, implicating the chondrocyte as the mediator of this matrix damage (Hollander et al 1995). The chondrocytes alter during OA with; (1) proliferation and cell death (2) changes in synthetic activity (3) matrix degradation (4) phenotypic modulation (5) osteophyte formation (Sandell and Aigner 2001).

Proliferation

A very low proliferative activity is observed in OA chondrocytes that is absent from normal cells (Poole 1997; Bush and Hall 2003). This proliferation is probably responsible for chondrocyte clustering seen in OA and may be due to better access to proliferative factors from the synovial fluid following fissuring/damage of the collagen network.

Apoptosis

Apoptosis is enhanced in OA, particularly close to the articular surface (Hashimoto et al 1998). However it is only seen in very small percentage of cells (<1%) with a small increase in empty lacunae (Aigner and Dudhia 1997), suggesting it will not contribute significantly to OA pathology (Sandell and Aigner 2001).

ECM binding is required for survival, with loss of binding leading to apoptosis (Anoikis). Loss of matrix components and their cell surface receptors, including collagen type II, integrins, hyaluronan and CD44, may therefore cause death (Aigner et al 2002b; Kuhn et al 2004). Mechanical injury can also induce cell death, with the magnitude and rate of application of force determining the outcome (Aigner et al 2002b; Kuhn et al 2004).

Mineral deposits are seen in zones of apoptosis, suggesting the cells may enter a differentiation state similar to that seen in the hypertrophic zone of cartilage. Apoptosis in chondrocytes can be blocked by caspase inhibitors. Although *in vitro* studies suggest that caspase inhibition does not always rescue cells from cell death, some experimental models demonstrate that caspase inhibition can lead to improved disease outcome (Kuhn et al (2004)).

Synthetic activity

Work has shown that a net loss of ECM components occurs at all stages in OA despite an early increase in synthesis. However these studies looked at the tissue as a whole. Individual cellular analysis revealed a loss of fixed charges occurs in the upper zones of OA cartilage, where the cells decreased matrix synthesis, while in the deeper zone where the ECM is still intact, cells remain activated (Aigner and Dudhia 1997). This increased synthesis of matrix may be an early attempt at repair, with collagen type II and aggrecan detected, a response which is lost in late OA (Poole et al 1991).

The collagen fibre network in cartilage consists of large crosslinked rigid structures that are difficult to repair or replace. The production of new collagen molecules fails to remodel and repair the tissue and overall tensile strength is reduced. Aggrecan has a faster turnover than collagen and so can be more easily replaced by newly synthesised molecules. PG changes occur initially at the cartilage surface, with chemical alterations and increased swelling (Thompson and Oegema 1979). Reduced ionic content and osmolality due to dilution of GAGs increases the cell volume by as much as 90% and will influence the balance between matrix synthesis and degradation (Bush and Hall 2003). In advanced OA the PG levels are significantly reduced as visualised by safranin O and alcian blue staining (Mankin et al 1971).

Matrix degradation

The major proinflammatory cytokines associated with cartilage loss are IL-1β and TNFα (Lotz et al 1995), with increased expression of both by OA chondrocytes (Towle et al 1997; Webb et al 1997). Increased expression of the type I IL-1 receptor (IL-1RI) in OA chondrocytes was associated with increased MMP production (Martel-Pelletier et al 1992). The p55 TNF receptor has been localised to chondrocytes at sites of focal loss of cartilage PGs (Webb et al 1997).

MMPs are found localised in regions of cartilage damage (Hembry et al 1995) and are elevated in the synovial fluid and cartilage from OA patients (Okada et al 1992). MMPs 1,2,3,7,8,13 and 14 are produced by chondrocytes (Sandell and Aigner 2001). Increased MMP3 (Stromelysin) is seen in early OA, with increased MMP13 and MMP2 in late stage OA. (Aigner et al 2002a; Aigner and McKenna 2002). There is a 10 fold increase in fibronectin in OA cartilage (Brown and Jones 1990). Proteolytic degradation of fibronectin produces fragments that are able to initiate degradation of aggrecan through autocrine/paracrine IL-1β synthesis that causes MMP induction (Arner and Tortorella 1995; Homandberg et al 1992; Roughley 2001), previously demonstrated in fibroblasts (Werb et al 1989). ADAM-TS4 and ADAM TS5 are present in OA cartilage and are responsible for aggrecan degradation. This generates novel termini (neoepitopes) that can be detected in the synovial fluid and cartilage of OA patients using specific antibodies, with the N-terminus of the molecule buried in the binding site cavity of the specific antibody (Hughes et al 1995; Mort et al 2003). Aggrecan neoepitopes are detected in vitro and in the synovial fluid of OA patients as a marker of tissue destruction, with an ADAM-TS inhibitor preventing aggrecan damage of cartilage in vitro (Malfait et al 2002).

Increased synthesis of matrix-degrading enzymes, including collagenase and aggrecanase, and decreased TIMP is seen in OA (Attur et al 2000; Lohmander et al 1999; Sandell and Aigner 2001). This decreased inhibition of MMPs will allow increased action in degrading the cartilage matrix.

Phenotypic modulation

Collagen types III, IIA and X are expressed in different zones of OA cartilage, suggesting alteration of the chondrocyte phenotype. No expression of collagen type I was detected in any zone of normal or OA cartilage. Collagen type IIA expression, a marker of dedifferentiation, is seen mainly in the upper-mid zone of OA cartilage (Aigner and McKenna 2002). Type X collagen synthesis (normally associated with calcification of the matrix through endochondral ossification) is found in the deep layer of cartilage in OA (von der Mark et al 1992). Hypertrophy is associated with apoptosis and partial calcification of the matrix. Any products of cell death will remain in the cartilage and contribute to pathologic cartilage degradation (Sandell and Aigner 2001).

The expression of cell adhesion molecules is altered in OA chondrocytes. An example of this is the upregulation of CD44 in deep zones of OA cartilage (Ostergaard et al 1997b). The increased expression of β1 integrin in OA cartilage has been shown in vitro and in vivo (Loeser et al 1995). The expression of α2, α4 and β2 integrins have been described in OA but not normal cartilage (Ostergaard et al 1998). These studies suggest that the normal chondrocyte-ECM interactions are interrupted in OA, which may play a role in the onset or progression of disease. Increased synthesis of fibronectin (Burton-Wurster et al 1986), type VI collagen (Swoboda et al 1998) and tensin (Salter 1993) have all been described during OA and will influence the way in which the cell senses mechanical signals. Alterations in collagen type VI, thought to link the chondrocyte to collagen type II in the pericellular matrix, have been described in OA (Aigner and McKenna 2002). This may impede proper cell-matrix interactions so will alter the way in which the cell senses strains and thus modulate the synthetic activity of the cells. Pericellular matrix is important in regulating the biomechanical environment of the chondrocyte, so alterations in OA may affect the magnitude and distribution of mechanical signals perceived by the chondrocytes (Alexopoulos et al 2003).

1.3 – Proinflammatory Cytokine signalling (Figure 1.3)

Cytokines and growth factors are important regulators of chondrocyte function. Gene deletion studies have revealed that few individual cytokines are absolutely essential for life or even for individual cellular functions, suggesting that one cytokine can compensate for the loss of another (Okazaki and Leonard 2002). Redundancy of cytokines may be explained partly by the observation that one or more receptor subunit may be utilised by different cytokines and also by regions of sequence homology in the cytoplasmic domains of some receptors which may underline common signalling pathways (Murakami et al 1991). Growth factors such as TGFβ, IGF-1 and the bone morphogenic proteins (BMP) are important in cartilage anabolism (Aigner et al 2002b). Anti-inflammatory cytokines such as IL-4 are expressed in OA as well as normal cartilage and can counteract the activities of proinflammatory cytokines, playing a chondroprotective role (Joosten et al 1997).

The proinflammatory cytokines IL-1β and TNFα are the principal mediators of cartilage catabolism through decreasing matrix synthesis and increasing its degradation (Goldring 2000). The production of iNOS and synthesis of NO has been suggested as the mechanism through which these cytokines mediate many of their effects. Interferon gamma (IFNγ) and Interleukin 6 (IL-6) are involved in the regulation of production of proinflammatory molecules including iNOS following IL-1β and TNFα stimulation of cells (de Vera et al 1996; Van de Loo et al 1997a). It is important, therefore, to understand the signalling pathways of these cytokines, the activation of iNOS and the effects mediated by NO.

1.3.1 - IL-1β

IL-1β is detected in OA where it influences the balance between matrix synthesis and degradation, leading to disease progression. IL-1β is activated by an IL-1 converting enzyme (ICE) (Slack et al 1993).

IL-1 receptor antagonist (IL-1ra) is a molecule that binds the IL-1 receptor but does not initiate intracellular signalling (Hannum et al 1990), so decreasing the number of receptors available for IL-1 binding. Type I (IL-1RI) and type II (IL-1RII) receptors exist. IL-1RII has a short cytoplasmic tail and appears to act as a control, binding IL-1 but not initiating any signal cascades and so preventing its activity. The large intracellular domain associated with IL-1RI, however, is involved in IL-1 mediated

signalling and biological responses (Bird et al 1992). The binding of IL-1 to the IL-1RI causes recruitment of the IL-1 receptor accessory protein (IL-1RacP) (Greenfeder et al 1995). The Toll/IL-1 receptor domain (TIR) on the cytoplasmic tail of both the IL-1RI and IL-1RacP acts as a binding site for MyD88 following a conformational alteration caused by IL-1 binding. MyD88 recruits the serine/threonine kinases, intracellular receptor for activated kinase (IRAK) 1, which associates with IL-1RacP, and IRAK2 that associates with IL-1RI (Slack et al 2000). This causes the IRAK phosphorylation that is required for activation of the transcription factor Nuclear Factor κB (NFκB). Hyperphosphorylation of IRAKs occurs during signalling, which leads to their degradation. Tollip (toll interacting protein) interacts with IRAKs which may allow its association with the IL-1R. TNF-receptor associated factor (TRAF) 6 activation and ubiquitination occurs following IRAK recruitment, interacting with the mitogen activated protein kinase (MAPK) TAK-1. TAK-1 is phosphorylated and ubiquitinated, causing activation rather than the classical degradation seen after ubiquitination. TAK1 can then activate the kinase responsible for phosphorylation of the NFκB inhibitor IκB, IKK-2, leading to release of NFκB for nuclear translocation and cellular signalling (IL-1β signalling reviewed in O'Neill 2002). TAK-1 can also activate p38 MAPK and c-Jun NH_2 terminal kinase (JNK), causing phosphorylation of transcription factors such as ATF-2 and the stabilisation of mRNA containing AU repeats such as TNFα and cyclooxygenase II (COXII) (Hua et al 2002). p38 MAPK inhibition decreased IL-1β induced collagen degradation in a cartilage culture model (Ridley et al 1997), and showed protection against cartilage destruction in the rat AIA model (Badger et al 2000).

1.3.2 - TNFα

TNFα is produced by chondrocytes in OA (Melchiorri et al 1998) and exists as a homotrimer in solution (Banner et al 1993). TNFα knockout mice develop more severe arthritis than wild-type mice as early chondrocyte apoptosis due to TNFα is prevented, allowing more time for the chondrocytes to produce the degradative enzymes responsible for cartilage breakdown (Butler et al 1997). The TNFα gene promoter contains a number of regulatory elements with increased TNFα production seen when IFNγ and IL-1β are added simultaneously implicating signal transducer and activator of transcription (STAT) involvement in addition to NFκB and AP-1 (Hua et al 2002). The 3'UTR of TNFα mRNA contains an octanucleotide AU repeat

sequence that is associated with mRNA degradation and interferes with translation (Hua et al 2002).

p55 and p75 are two TNFα receptors present in cartilage, although not both on the same cells. TNFα can self-regulate through the induction of cleavage of its own receptors which are capable of neutralising TNFα. This response, however, is disrupted in OA (Westacott et al 2000). The p55 receptor cytoplasmic domain contains potential phosphorylation sites for the cAMP dependent protein kinase, protein kinase C (PKC) and tyrosine kinases that are not seen on p75. A serine rich domain on p75, however, constitutively undergoes phosphorylation. The crystal structure reveals that three receptor molecules bind symmetrically to one TNFα trimer (Banner et al 1993). Both receptors bind TNFα with equally high affinity and transduce signals independent of each other. After TNFα binds its receptor it is quickly internalised and degraded, with TNFα receptor expression regulated by a number of factors including IL-1, IL-4, IL-6, TNFα, and PKC. Both receptors can activate NFκB, although most of the biological effects of TNFα are mediated via the p55 receptor (Nicola NA).

TNFα reduces the activity of the collagen type II and link protein promoters via MEK1/2 and NFκB, rather than through p38 or JNK in rat articular chondrocytes (Seguin and Bernier 2003). However, in another study NFκB and AP-1 were activated by TNFα via p38 and JNK in human astrocytes, suggesting cytokine signalling is a complex process (Hua et al 2002). The binding of upstream regulators of MAPKs (MEKKs) to regulatory proteins can alter specificity, seen in TNFα signalling where Gck links MEKK1 to JNK activation, whilst Tax association allows preferential NFκB stimulation (Shi and Kehrl 1997; Yin et al 1998; Yuasa et al 1998).

1.3.3 - IL-6

IL-1β, TNFα and IFNγ can increase IL-6 in fibroblasts (Akira et al 1993). Transcriptional control elements include NFκB, nuclear factor IL-6 (NF-IL-6) and an AP-1 site. Fluid induced shear using a cone viscometer for 1-48 hours induced IL-6 production in normal and OA human chondrocytes. This had little effect on PG synthesis and degradation. It does, however, modulate the effects of growth factors and pro-inflammatory mediators (Mohtai et al 1996). IL-6 heterogeneity is seen

through N and O linked glycosylation and phosphorylation, which could explain any differences in its observed activities (Nicola NA). sIL-6Rα was found to potentiate the inhibitory effect of IL-6 on PG synthesis, but this effect is moderate compared to IL-1β (Guerne et al 1999; Silacci et al 1998). IL-6 knockout mice showed decreased inflammatory cell infiltration but elevated PG loss during the acute phase of zymosan induced arthritis (Van de Loo et al 1997b).

The IL-6 receptor complex is made up of two non-binding signal transducing gp130 molecules and a single low affinity IL-6 alpha chain receptor (Murakami et al 1993), yielding a high affinity receptor capable of signal transduction (Hibi et al 1990). Signalling occurs as homodimerised gp130 associates with a tyrosine kinase, causing gp130 phosphorylation (Murakami et al 1993). The tyrosine kinase is likely to be JAK1, JAK2 or Tyk2 as all bind to gp130 and are activated after gp130 stimulation with IL-6 (Stahl et al 1994). STAT3 activation by tyrosine kinases attached to gp130 allows its translocation to the nucleus, which due to its large size requires a nuclear import receptor. MEKK1 and SEK can enhance the transcriptional activity of STAT3 by further phosphorylation (Schuringa et al 2000). IL-6 is also involved in activation of ERK1/2, p38 and JNK as well as PI3kinase causing protein kinase B (PKB) activation of proapoptotic factors. Several control mechanisms exist, including the recruitment of SHP2 phosphatase to activated gp130 that can inhibit JAK and STAT activation. Protein inhibitors of activated STATs (PIAS) are also important regulators of the JAK/STAT pathway, with PIAS3 inhibiting STAT3 activation due to IL-6 and PIAS1 preventing IFNγ induced STAT1 signalling. SOCS (suppressors of cytokine signalling) produced by IL-6 via the JAK/STAT pathway inhibit STAT mediated signal transduction and so act as classical feedback inhibitors (IL-6 signalling reviewed in (Nicola NA).

STAT binding sites are often in close proximity to binding sites for other transcription factors, such as NF-IL-6 (Kordula and Travis 1996), NFκB (Brown et al 1995) and AP-1 (Zhang et al 1999). This suggests a mechanism by which synergy can occur when more than one cytokine is present.

1.3.4 - IFNγ

Increased IFNγ is seen in RA and OA (Westacott et al 1990). There is synergistic activity of IFNγ with other cytokines such as IL-1β, upregulating pro-inflammatory factors such as NO, IL-6 and prostaglandin E_2 (PGE_2), and decreasing PGs. IFNγ also shows some anti-inflammatory effects where it synergistically increases IL-1ra production, so controlling IL-1 signalling, as well as decreasing IL-8 and stromelysin (Henrotin et al 2000). It may be that different concentrations of IFNγ have different effects, or it could be that different subpopulations of chondrocytes within the joint differ in their responsiveness to cytokines.

The IFNγ molecule is active in a dimeric conformation (Farrar and Schreiber 1993) and the receptor contains both an alpha and a beta subunit. IFNγ binds two alpha receptors and causes alpha subunit dimerisation (Fountoulakis et al 1992), bringing the two receptors together. The alpha subunit cytoplasmic domain contains a sequence that associates with a kinase that is likely to be JAK1 or JAK2 as these are required for IFNγ induced cellular responses (Watling et al 1993). The beta subunit has a similar JAK site (Murakami et al 1991), binding either JAK1 or JAK2. The close proximity of the receptors following IFNγ binding brings the JAKs together, causing their phosphorylation and activation as well as alpha chain tyrosine phosphorylation that creates a p91 binding site close to the activated tyrosine kinase. p91 is a gamma-activated factor which migrates from the cytoplasm to the nucleus where it binds to the gamma-activated sequence (GAS) of IFNγ responsive promoters (Shuai et al 1992). p91 binds and is activated by phosphorylation, dissociating from the IFNγ receptor and associating with other activated p91 or activated transcription factors causing nuclear translocation and gene transcription (Shuai et al 1992). The identification of STAT1 docking sites on the IFNγ receptor causing STAT1 phosphorylation, dimerisation and subsequent activation through further phosphorylation has also been implicated in IFNγ signalling. Activated STAT-1 then binds DNA and affects gene transcription (Nicola NA).

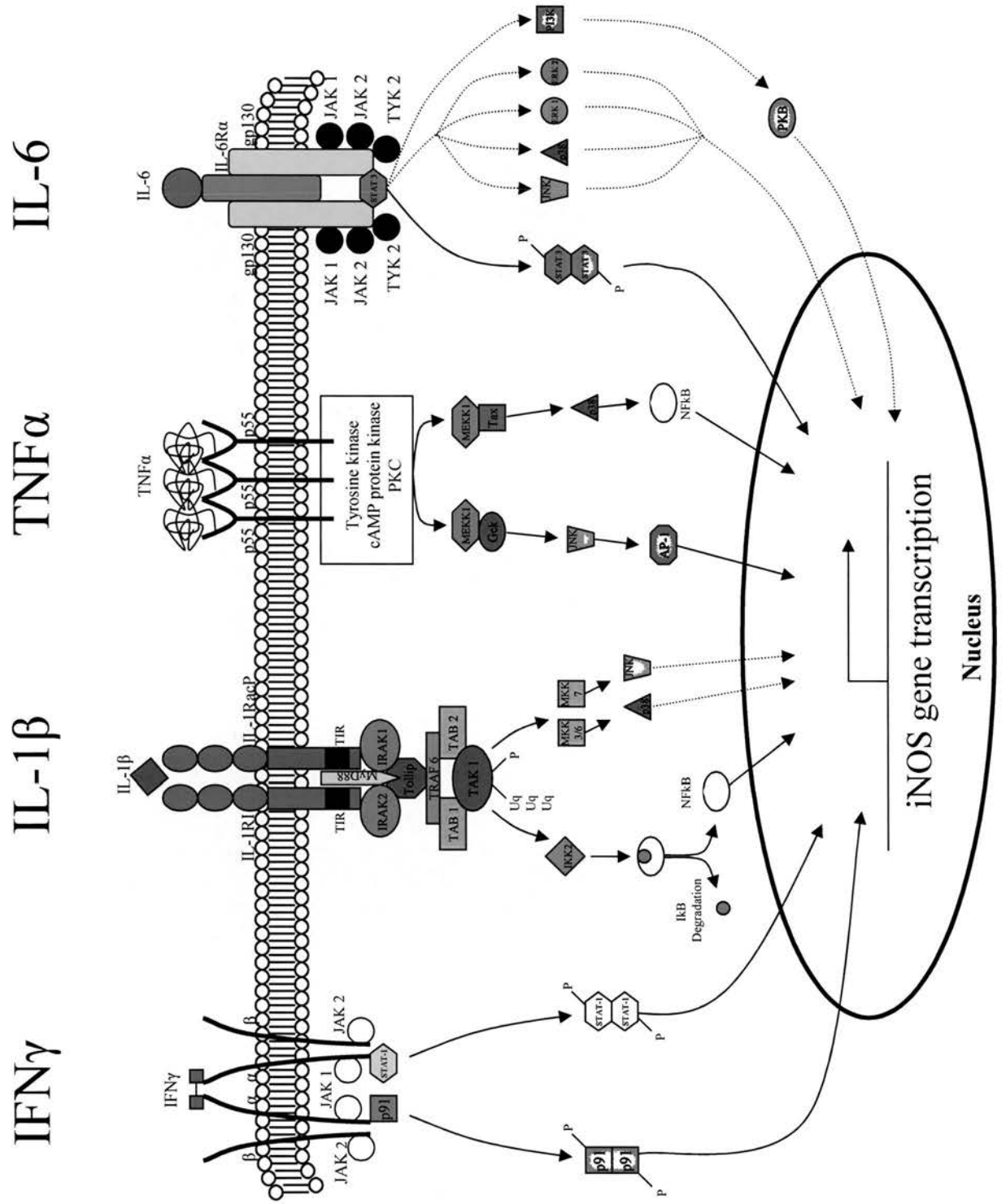


Figure 1.3 – Proinflammatory cytokine signalling. IL-1β, TNFα, IL-6 and IFNγ all attach to different receptors and initiate different intracellular signalling cascades. Each of these cytokines activate transcription factors that can influence iNOS gene transcription (complete arrows entering nucleus). Several other molecules are also activated that may influence iNOS gene transcription (dashed arrows entering nucleus).

1.3.5 - Nitric Oxide Synthase (NOS)

NOS catalyses the oxidation of arginine (arg) to NO and citrulline. There are three genetically distinct NOS isoforms, neuronal NOS (nNOS or type I), endothelial NOS (eNOS or type III) and inducible NOS (iNOS or type II). These were initially identified in neurons, endothelial cells and macrophages respectively (Bredt and Snyder 1990; Pollock et al 1991; Xie et al 1992). The NOS contain both an N-terminal oxygenase and a C-terminal reductase domain. The oxygenase domain binds haem, tetrahydrobiopterin (BH_4) and arg, whilst Flavin adenine dinucleotide (FAD), Flavin mononucleotide (FMN), Nicotinamide adenine dinucleotide phosphate (NADPH) and Calmodulin (CaM) are associated with the reductase domain (Ghosh and Stuehr 1995; Richards and Marletta 1994). The enzyme is activated upon homodimerization that requires the presence of the haem group, as well as BH_4 and arg for stabilisation (Klatt et al 1995). Zinc tetrathiolate has been located at the dimer interface, tetrahedrally binding to two cysteines from each subunit and is also involved in dimer stability (Chen et al 1995; Fischmann et al 1999).

The active NOS enzymes gain electrons from NADPH and transport them through FAD and FMN to the haem group (Adak et al 1999) of the other NOS enzyme in the dimer (Siddhanta et al 1996) (Figure 1.4). Arg is closely associated with, but not bound to, the haem group and can be oxidised to citrulline and NO (Crane et al 1998).

Each of the NOS isoforms has a different activity, with turnover numbers for NO synthesis of approximately $200min^{-1}$, $100min^{-1}$ and $20min^{-1}$ for iNOS, nNOS and eNOS respectively (Roman et al 2002). The binding of CaM increases the rate of electron transfer from NADPH through the flavins to the haem centre through a conformational change (Abu-Soud et al 1994; Craig et al 2002). An autoinhibitory loop prevents CaM binding in low calcium (Ca^{2+}) concentrations in both eNOS and nNOS (Figure 1.5), so regulating the activity of the enzymes (Salerno et al 1997). C-terminal tails of 20-42 amino acids long have been identified on the NOS isoforms and are suggested to both protect the flavins from external oxidation and inhibit the electron flow between them (Roman et al 2000a; Roman et al 2000b; Roman et al 2002) (see Figure 1.5). The inhibitory function of the tail may be important in iNOS in preventing excessive electron delivery to the haem, which would result in inactivity of the enzyme (Adak and Stuehr 2001).

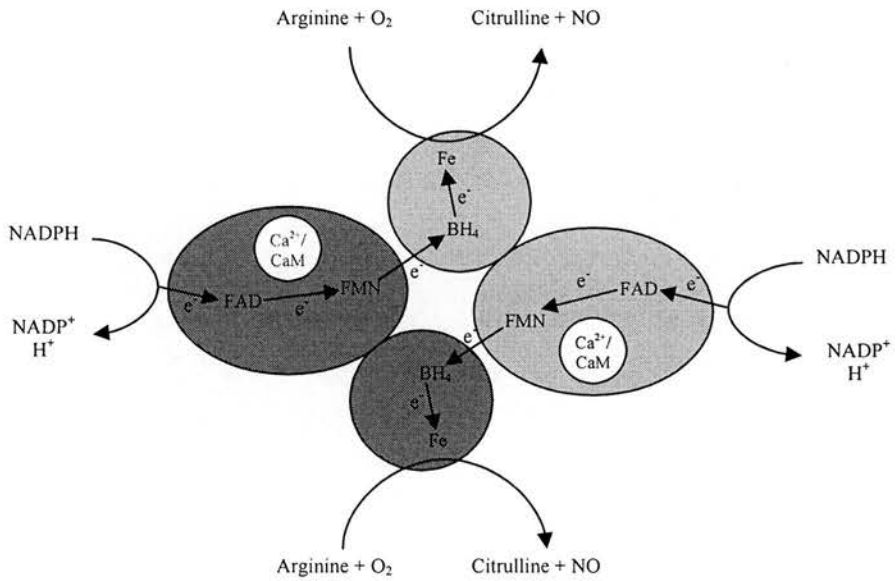


Figure 1.4 - Electron flow and reaction catalysed by NOS
Within the NOS homodimer the electrons flow from NADPH → FAD → FMN in the reductase domain of one monomer to the BH_4 and Haem in the oxygenase domain of the other monomer. The BH_4 and Haem within the active site catalyse the reaction of oxygen with arginine, generating citrulline and NO as products.The presence of Ca2+/CaM is required for electron flow through the reductase domain.

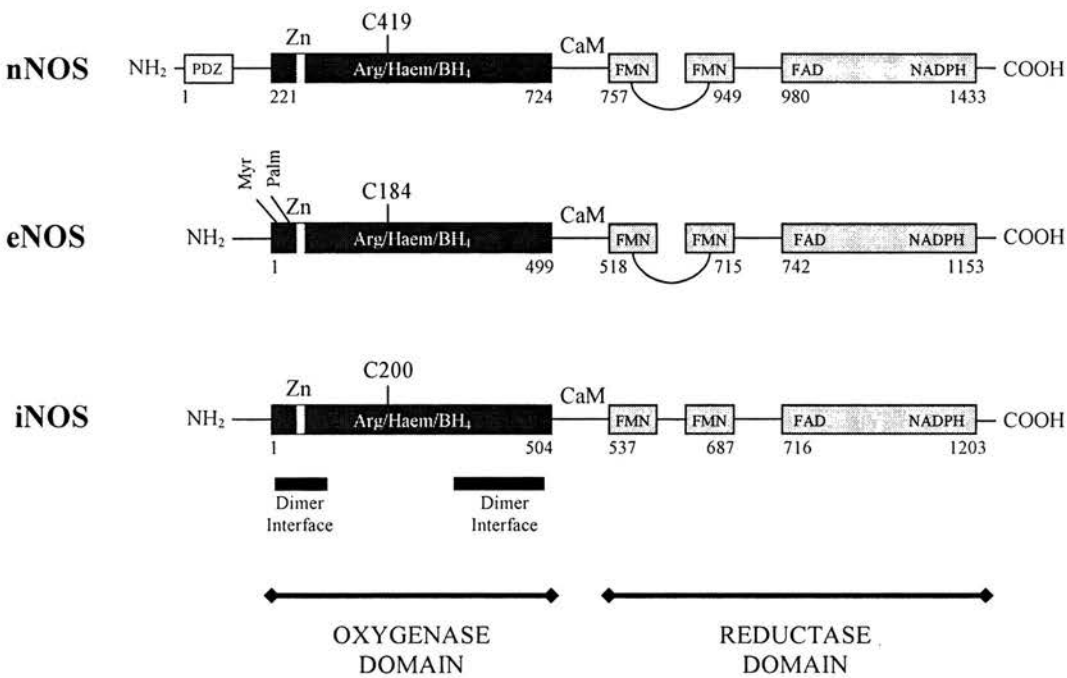


Figure 1.5 - Domain structure of human nNOS, eNOS and iNOS
Oxygenase, reductase and PDZ domains are denoted by solid boxes with the amino acid residue number at the start/end of each domain. The cysteine residue which ligates the heme, location of Zinc (Zn) ligating cysteines and the Calmodulin (CaM) site is indicated in each isoform. The autoinhibitory loop within the FMN regions of nNOS and eNOS are shown as are the dimer interface in the oxygenase domain. Myristoylation (Myr) and palmitoylation (Palm) sites on eNOS are shown. Adapted from Alderton et al (2001).

eNOS

NO was originally described as an endothelial derived relaxing factor. In the endothelium NO is produced in picomolar amounts in response to receptor stimulation by molecules such as acetylcholine and bradykinin, and to sheer stress (Farrell and Blake 1996). The production of NO has a biphasic response (Mullershausen et al 2001). NO initially influences basal vascular tone by diffusion to adjacent vascular smooth muscle cells and activation of the haem containing enzyme soluble guanylyl cyclase. This leads to increased cGMP levels resulting in activation of different cGMP-dependent effector proteins such as protein kinases (Hofmann et al 2000), phosphodiesterases (Juilfs et al 1999) and ion channels (Biel et al 1999). The relaxation of vascular smooth muscle leading to vasodilation occurs at this stage. However, the second stage is activation of phosphodiesterase 5 by NO, which leads to a rapid decrease in cGMP causing NO induced desensitisation of the cGMP response (Mullershausen et al 2001).

Phosphorylation of the reductase domain of eNOS has been shown at four sites, Thr 495, Ser 617, Ser 635 and Ser 1177 (Chen et al 1999; Michell et al 2001), each influencing enzyme activity. PKC phosphorylates eNOS at Thr 495, negatively regulating eNOS activity (Matsubara et al 2003), while PKB phosphorylates eNOS at Ser 1177 stimulating its activity (Ming et al 2002).

nNOS

NO synthesis is seen throughout the brain (Farrell and Blake 1996). Coupled to the NMDA receptor, nNOS is activated by the influx of calcium associated with glutamate activation. This can lead to neurotoxicity but may also be neuroprotective depending upon the redox state of NO. NO could potentially act as a retrograde synaptic messenger, where modulation of synaptic transmission may cause long term potentiation type responses that are implicated in learning and memory. nNOS can also mediate vasodilation; so coupling neural activity to local increases in blood flow. There is also a role for NO in pain mechanisms and it may be both pro- or anti-nociceptive at different sites (Farrell and Blake 1996).

Phosphorylation of the inhibitory loop of nNOS has also been described but, unlike in eNOS, this decreases both electron transfer and NO production (Hayashi et al 1999). The kinases implicated in nNOS phosphorylation are different to those seen for eNOS. Calcium/calmodulin dependent protein kinase II (CaM Kinase II)

phosphorylates nNOS at Ser 847, which decreases nNOS activity (Rameau et al 2004). PKA and PKG have also been implicated in nNOS phosphorylation and subsequent downregulation of activity (Dinerman et al 1994).
Eliasson et al (1997) reported more than ten differentially spliced nNOS transcripts, producing several different proteins.

iNOS

Expression of the iNOS gene is an important part of the immune response to infection, with macrophage phagocytosis leading to iNOS induction in humans (Watkins et al 1997) and LPS shown to induce iNOS in rodent macrophages (Nathan and Xie 1994). Indeed, in knockout (KO) mice studies infection cannot be appropriately cleared, leading to chronic infection and death (Mashimo and Goyal 1999). The much reduced inflammatory response is well demonstrated following injection of iNOS KO mice with lipopolysaccharide (LPS), where it prevents the death seen in the wild type (WT) mice (Wei et al 1995). Overexpression of iNOS is seen in human diseases such as OA and rheumatoid arthritis (RA), where expression is induced by exposure to inflammatory cytokines such as IL-1β, TNFα and IFNγ (Kahle et al 1992; Melchiorri et al 1998; Westacott et al 1990).

Due to the role of iNOS in host immunity and the pathophysiology associated with its production during disease, it is not surprising that the regulation of production is complex.

iNOS Transcriptional Regulation

The human iNOS (hiNOS) promoter is a sixteen kilobase region at the 5' untranslated region (UTR) of the hiNOS gene that contains several sequences coding for both positive and negative regulatory elements (Figure 1.6).

Cytokine responsive elements upstream of -3.8kb are required for iNOS transcriptional activation. Five NFκB binding sites from –4.7kb to –7.2kb were identified, with the site at –5.8kb required for promoter activity and the others cooperatively increasing iNOS gene transcription (de Vera et al 1996; Taylor et al 1998). A negative regulatory element (NRE) identified by Feng et al (2002) at –6.7kb binds constitutively expressed NFκB repressing factor (NRF). This interaction is implicated in control of promoter activity regulating basal iNOS expression.

The investigation of IFNγ effects on hiNOS gene transcription focussed on the JAK mediated STAT 1 binding to the hiNOS promoter. IFNγ (Dalton et al 1993), IFNγ receptor (Kamijo et al 1993) and STAT-1 (Meraz et al 1996) KO mice are defective in iNOS induction by cytokines. The previously identified –5.2kb NFκB site was shown to bind STAT-1, with the –5.8kb site shown to facilitate both STAT-1 and NFκB binding (Ganster et al 2001).

Marks-Konczalik et al (1998) identified two further NFκB sites and two AP-1 sites in the first 8.3kb of the promoter that are required for transcription. The AP-1 transcription factor subunits are Jun D and Fra 2 while both Rel A/Rel A and RelA/p50 subunit dimers make up NFκB. The role of AP-1 in iNOS expression is controversial, it is likely that different dimers of AP-1 factors will bind to activator or repressor sites. Indeed, an AP-1 like site was identified by Pance et al (2002) that caused repression of hiNOS transcription.

This transcription factor network is further complicated by other transcriptional control proteins including NF-IL-6, which are known to interact with NFκB and have sites on the hiNOS promoter (LeClair et al 1992; Marks-Konczalik et al 1998). It is therefore likely that complex mechanisms involving many transcription factor combinations may actually regulate the iNOS gene.

This is supported by the interactions between transcription factors involved in iNOS induction. IFNγ-induced IFN regulatory factor (IRF-1) and TNFα induced NFκB interact while binding the hiNOS promoter in stimulated macrophages (Saura et al 1999). It has been suggested that this interaction physically bends the hiNOS promoter DNA and thus may explain the mechanism by which IFNγ increases iNOS transcription. Histone deacetylase (HDAC) 2 interacts with the NFκB subunit p65, with increased HDAC 2 causing increased cytokine-induced iNOS. This indicates that hyperacylation may act by limiting the functional efficacy of NFκB, so regulating iNOS (Yu et al 2002a).

STAT-3 also binds the p65 subunit of NFκB, acting as a dominant negative inhibitor of NFκB activity, causing the indirect suppression of cytokine-induced iNOS (Yu et al 2002b).

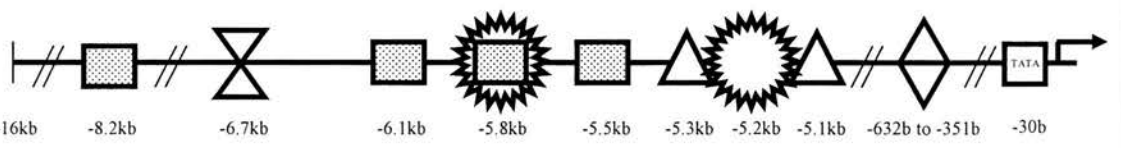
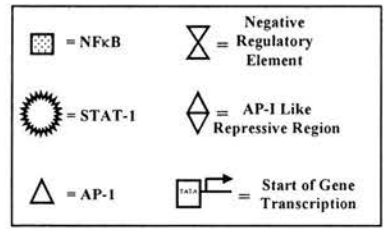


Figure 1.6 - Human iNOS gene promoter sequence
Mutational and deletional studies of the human iNOS 5'UTR have identified several sites specific for transcription factor binding (NFκB, STAT-1 and AP-1) involved in cytokine mediated iNOS induction. This region also contains negative regulatory regions (AP-1-like site and Negative regulatory element recognised by NFκB repressing factor) that inhibit iNOS induction. The section from –8.2kb to –16kb is known to affect iNOS transcription but the specific elements within this region have yet to be characterised.



iNOS Post-Transcriptional Regulation

Four AUUUA and one AUUUUA motifs have been identified in the 3'UTR of hiNOS, which are known to destabilise mRNA (Rodriguez-Pascual et al 2000). A protein, HuR, binds to the AU rich elements of the iNOS 3'UTR. Inhibition of HuR can reduce cytokine-induced iNOS mRNA whereas overexpression potentiates cytokine-induced iNOS expression (Rodriguez-Pascual et al 2000). Calcium has also been shown to inhibit IL-1β induced NO by reducing iNOS mRNA stability (Geng and Lotz 1995).

Alternative splicing of the hiNOS gene occurs, with deletions of exon 8 and exon 9 described. Eissa et al (1998) showed that these deletions do not affect the production

of iNOS protein, but do inhibit its activity by preventing dimerisation. iNOS splice variants with deletions in haem and FMN domain have also been detected (Eissa et al 1996).

Albakri and Stuehr (1996) showed that NO can interact with cellular haem, preventing its incorporation into the iNOS enzyme. NO can also inactivate the enzyme through the formation of a stable ferrous nitrosyl complex with the haem in the enzyme (Cooper 1999). An NO donor (SNAP) inhibited dimerisation of iNOS monomers without affecting haem release (Chen et al 2002). This inhibition of iNOS activity by NO itself indicates a classic negative regulatory mechanism by which NO production can control its own levels.

iNOS phosphorylation has been reported, however the role this has has not been further investigated with the suggestion that it may play a role in the control of subcellular localisation (Pan et al 1996).

iNOS Protein-Protein Interactions

There are several proteins that directly interact with iNOS influencing its expression (Zhang et al 2003). Kalirin and NOS-associated protein 110kDa (NAP110) bind iNOS monomers, preventing homodimerisation and so decreasing activity (Ratovitski et al 1999a; Ratovitski et al 1999b). Caveolin-1 binds iNOS in a similar way to that described for both eNOS and nNOS (Felley-Bosco et al 2000), inactivating the enzyme. EBP50 is a PDZ domain-containing scaffolding protein that binds iNOS and interacts with other PDZ-containing proteins such as ion channels. This can localise iNOS to protein complexes associated with the actin cytoskeleton (Zhang et al 2003). Rac 1 and Rac 2 are members of the Rac family of Rho-like GTPases and interact with iNOS (Kone et al 2003; Kuncewicz et al 2001) influencing its intracellular localisation and activity.

iNOS Proteasomal degradation

Musial and Eissa (2001) showed the two phase regulation of iNOS by the 26s proteasome degradation pathway. The activation of iNOS by IκBα degradation and subsequent NFκB translocation and activation occurs initially, followed by the

degradation of the iNOS protein itself. The degradation of iNOS is mediated by binding to Caveolin-1 in human colon carcinoma cells (Felley-Bosco et al 2000).

Other iNOS Regulatory Molecules

The presence of other molecules within the cell also influences the activity of iNOS. Arginase is an enzyme that competes with iNOS for the substrate arg. The presence of arginase can deplete cellular arg stores and so regulate the activity of iNOS (Bogdan 2001). The availability of the substrate arg will control the rate of synthesis of NO, and some cells can generate arg from citrulline, so using citrulline to produce NO (Hattori et al 1994; Nussler et al 1994). In endothelial cells eNOS localises with the arg transporter CAT-1 and arg-generating enzymes in caveolae (Flam et al 2001). Glutathione (GSH) is the major thiol in cells and plays an important role as an antioxidant or detoxicant agent against reactive oxygen species (ROS) and reactive nitrogen species (RNS). This is likely to be the mechanism by which iNOS producing cells defend themselves against NO. The NO bound to GSH can, however, be passed to other molecules suggesting GSH may be acting as a store that maintains the biological activity of NO (Kroncke et al 2001; Meister and Anderson 1983). The saturation of GSH stores allows other ROS and subsequent NO released to have effects on other cellular proteins (Coleman 2001; Wink et al 1996a). Haem Oxygenase 1 (HO-1) acts as an anti-inflammatory enzyme as it decreases iNOS and COX-2 production in both migrating leukocytes and in macrophages (Coleman 2001). HO-1 expression is also induced by oxidised phospholipids, produced following ROS reaction with phospholipids (Kronke 2003). Superoxide dismutase (SOD) is an enzyme that scavenges oxygen free radicals, preventing lipid peroxidation, decreasing iNOS activity and decreasing IL-1β and TNFα release (Zhang and Rosenberg 2002). Alteration of the pH has been shown to influence NOS activity through variations of calcium sensitivity. A shift from pH 7.2 to 6.8 led to a decrease in NOS activity (Conte 2003).

1.3.6 - Effects of NO

NO is a molecule with a short half-life and low molecular weight and with high diffusibility and lipid solubility, which confers upon it the properties to act as a signalling molecule. The half-life of NO is increased at lower concentrations from seconds to minutes/hours (Wink et al 1996b). The impact of NO on cell function or death are complex and often appear contradictory. It can be toxic/antitoxic, apoptotic/antiapoptotic, antioxidant/compromise cellular redox state via oxidation of thiols like GSH, inhibit/activate signalling pathways, inhibit/activate gene transcription (Kroncke et al 2000).

NO can modulate cells by various mechanisms including:-

1) Binding to haem
2) Reaction with free thiols
3) Reaction with superoxide anion
4) Nitrosylation of target proteins
5) ADP-ribosylation of proteins (Studer et al 1999).

The interaction with transition metals and thiol groups located at either allosteric or active sites are two methods of NO control that can affect many molecules within the cell (Table 1.1). This allows NO to influence the activity of enzymes involved in many cellular functions (Coleman 2001).

Reaction with free thiols leads to the production of molecules with similar biological activity to NO but a longer half-life, the s-nitrosothiols. Both nitrosylation and ADP-ribosylation of proteins by NO can modulate their function or behaviour. Reaction of NO with ROS, such as the superoxide radical (O_2^-), increases the range of biological activity of NO through the production of molecules such as peroxynitrite ($ONOO^-$). NO has been suggested to be a regulator of LPS activated signalling pathways, whereas IL-1β activated signal transduction is more sensitive to peroxynitrite (Mathy-Hartert et al 2003).

Cysteines are important in DNA binding on several transcription factors, including c-fos, c-jun, AP-1 and NFκB (Hentze et al 1989; Schreck et al 1991). This allows NO to cause nitrosylation and function modification, suggested to act as redox-sulphhydryl switches that directly regulate gene expression (Hentze et al 1989).

Both activation of NFκB and inhibition of NFκB by NO has been described (Coleman 2001). The production of NO derivatives can explain this difference, with peroxynitrite promoting NFκB activation through sustaining p65 nuclear translocation, whereas s-nitrocysteine inhibits NFκB activity (Clancy et al 2004).

NO can also influence other inflammatory mediators in both a positive and negative way, increasing TNFα release by leukocytes through interaction with an NO responsive element in the TNFα gene promoter (Lander et al 1993) Coleman 2001). NO can also decrease the production of PGE_2, IL-6 and IL-8 (Coleman 2001; Henrotin et al 1998).

The list of functions and effects of NO continues to grow as more is learned about this molecule in different systems. Potential involvement of NO in the OA disease process is described through effects on inflammation and cartilage matrix homeostasis.

REACTION SITE	**LOCATION**			
	Membrane	Cytosolic (Including compartments)	Nuclear	Extracellular
Thiol	NMDA receptor	GAPDH	AP-1	Glutathione
	K_{ca}^+ channel	Actin	NFκB	Albumin
	NADPH oxidase	Glutathione		
	G proteins/p21ras			
	Protein kinase C			
	Adenylyl cyclase			
Metal		Guanylate cyclase	SoxRS	
		Hemoglobin		
		Aconitase/IRE-BP		
		Oxidoreductases complex I and II		
		Cyclooxygenases		
		Cytochrome P450s		
		NO synthases		
Unknown		Ribonuclease Reductase	CREB	

Table 1.1 – Molecules affected by NO interaction (adapted from Stamler 1994)

Effects of NO in Cartilage (Table 1.2)

Increased iNOS and NO are detected in OA cartilage and synovium (Grabowski et al 1997). The detection of lipid peroxidation products (Kavanaugh 1998), nitrite (Spreng et al 2001) and nitrated type II collagen peptide (Henrotin et al 1990) in chondrocytes implicate NO in the disease process. The increase of nitrotyrosine and nitrated proteins in cartilage of arthritis patients demonstrates direct involvement of NO (Fukuda et al 2000; Loeser et al 2002). The increased range of biological activity after reaction of NO with ROS is implicated in cartilage degradation (Henrotin et al 2003).

IL-1β mediates a decrease in collagen type II directly by an NO independent action on collagen II mRNA levels. An NO dependent mechanism also exists where the inhibition of the enzyme prolyl hydroxylase by NO leads to underhydroxylation of collagen monomers, causing their degradation (Cao et al 1997). NO has also been implicated in the inhibition of PG synthesis by IL-1β, which will be discussed in more detail later in this Chapter and in Chapter 5 (Taskiran et al 1994).

IL-1β causes increased MMP production in chondrocytes (Sasaki et al 1998; Tamura et al 1996). NO has been implicated in MMP synthesis, as iNOS inhibition decreases IL-1-induced gelatinase, collagenase, stromelysin (Murrell et al 1995; Stadler et al 1991) and MMP9 (Sasaki et al 1998) gene expression.

iNOS knockout mice, however, show increased susceptibility to adjuvant-induced arthritis and septic arthritis, which indicates NO may have anti-inflammatory effects that impede OA development (Gilkeson et al 1997; McInnes et al 1998; Veihelmann et al 2001). NOS inhibitors have been shown to increase MMP activity associated with IL-1β stimulation (Stefanovic-Racic et al 1997). Bird et al (2000) describe a catabolic role for NO in decreasing PG synthesis, but a protective role in decreasing PG degradation through regulation of aggrecanase activity. The inhibition of PGE_2 by NO has been shown using NOS inhibitors (Henrotin et al 1998; Mathy-Hartert et al 2002). This has been suggested to be the mechanism by which NO could inhibit MMP activity (Stadler et al 1991). Despite its action on PGE_2 no effect is seen of NO on COXII expression, although this has been shown to be affected by peroxynitrite (Mathy-Hartert et al 2003).

NO reacts directly with iron-sulphur enzymes as well as via reaction with the superoxide radical causing peroxynitrite production. This can lead to the inhibition or inactivation of enzymes such as Aconitase (rate limiting step of the citric acid cycle), Complexes I+II in the mitochondrial electron transport chain, and Ribonuclease reductase involved in the synthesis of deoxyribonucleosides required for cell replication (Ouellet et al 2002). This allows NO to cause decreased aerobic energy metabolism (Stadler et al 1991), decreased DNA replication (Lepoivre et al 1990) and can lead to cytotoxicity (Hibbs et al 1987).

ADP-ribosylation of actin by NO prevents its polymerisation (Clancy et al 1995). The actin cytoskeleton is an important structure required for a number of cellular functions including intracellular signalling, chondrocyte migration and attachment to the ECM, the disruption of which may prevent chondrocyte migration into damaged areas so impairing cartilage repair (Frenkel et al 1996). Auto-ADP ribosylation of GAPDH, an enzyme involved in glycolysis and ATP generation, may also be mediated by NO, causing enzyme inhibition and so affecting energy production (Dimmeler et al 1992; Duman et al 1991).

The induction of lipid peroxidation by the hydroxyl radical and peroxynitrite may contribute to tissue damage (Lipton et al 1993), affecting cells by influencing membrane bound integrin-matrix interactions, activating MMPs, and generating other ROS and further metabolites in the pericellular environment (Tiku et al 2000).

NO is involved in preventing negative feedback control of IL-1β. Inhibition of NO leads to increases in IL-1β induced production of the IL-1β inhibitor IL-1ra (Pelletier et al 1996). Vuolteenaho et al (2003) also demonstrated that IL-1ra production by OA cartilage pieces was increased by IL-1β and further increased by the iNOS inhibitor 1400W, suggesting NO actually inhibits IL-1β negative feedback.

NO and Apoptosis

Fas is expressed on the surface of cultured chondrocytes from normal and OA cartilage, with binding of Fas ligand initiating apoptosis. The intact ECM is likely to protect chondrocytes from Fas ligand, while in damaged cartilage they will be exposed to it, which will increase apoptosis (Kuhn et al 2004).

Evidence for cell death as a consequence of endogenous NO has not been reported. NO released by sodium nitroprusside (SNP), however, induced apoptosis in cultured human chondrocytes (Blanco et al 1995). In rabbit chondrocytes SNP induced p38 dependent cell death via caspase 3 (Kim et al 2002). SNP increased caspase 3 activity in human OA chondrocytes, and caspase 3 inhibition blocked apoptosis by NO in one study (Notoya et al 2000). Disruption of the zinc-finger motif in DNA repair enzymes by NO increases apoptosis (Kroncke and Carlberg, 2000; Kroncke et al, 2002). DNA itself can also be oxidatively damaged by peroxynitrite, which could influence cell survival and potentially contribute to joint pathology (Juedes and Wogan 1996). In a canine model of OA the iNOS inhibitor L-NIL reduced TUNEL positive cells, but it is unclear if this was a direct effect of NO inhibition or due to protective effects against cartilage degradation (Pelletier et al 2000). In human chondrocytes the effects of NO donors on cell death are age dependent. The increased oxidised:reduced glutathione ratio in older chondrocytes may cause increased susceptibility to oxidant stress and explain why older donors are more susceptible to cell death due to NO (Carlo and Loeser 2003). Disruption of the actin cytoskeleton inhibits NO induced apoptosis through modulation of signalling. An example of this is the increased differentiation and decreased NO induced apoptosis seen following IGF-1 mediated cytoskeletal disruption (Lo and Kim 2004).

Carlo and Loeser (2002) reported that NO is not by itself an inducer of chondrocyte apoptosis, requiring other ROS. NO has been shown to have some protective effects and NO induced by adenoviral transfer of the iNOS gene to rabbit chondrocytes did not cause cell death (Studer et al 1999). This protective effect of NO may be mediated through the s-nitosylation of cysteine residues in the catalytic centre of caspases (Kolb 2000).

Van't Hof et al (2000) showed the colocalisation of apoptosis and iNOS production in cartilage, the iNOS inhibitor L-NMMA inhibited, and the NO donor SNAP induced this apoptosis. The iNOS knockout mouse highlighted the role of NO in apoptosis as

decreased nitrite, increased bone mineral density and little apoptosis was seen compared to the wild type in a model of inflammation mediated osteoporosis (Armour et al 2001).

OA progression	OA inhibition
↓ Collagen and PG synthesis	↓ MMP activity
↑ MMP synthesis	↓ PGE_2
↓ cellular energy production	↓ Inflammatory cytokine production
Signalling complexes disrupted due to ↓ actin polymerisation and lipid peroxidation	↓ NADPH oxidase activity
DNA damage → cell death	↓ iNOS activity
↓ IL-1 receptor antagonist	
↓ IGF-1 signalling	
Role in chondrocyte apoptosis	

Table 1.2 – Opposing effects of NO on OA progression

1.3.7 - iNOS Inhibition

Positive effect of iNOS inhibition

L-NMA treatment of rat adjuvant induced arthritis (AIA) administered prophylactically showed decreased disease as measured by paw swelling, plasma fibrinogen levels and urinary nitrite/nitrate (Stefanovic-Racic et al 1995). Delaying administration or the use of other inhibitors such as AG and L-NAME, were not effective. Connor et al (1995) used rat AIA to show that L-NIL and AG led to the inhibition of disease progression. Two studies by Pelletier et al (1999 and 2000) using the dog anterior cruciate ligament (ACL) transection model of OA measured the effects of L-NIL treatment. A dose dependent decrease in osteophyte size, macroscopic and histologic lesions, MMP activity and apoptosis associated with decreased caspase-3 was seen. Addition of an oxygen radical inhibitor decreased progression of rat AIA (Sakai et al 1999). Cuzzocrea et al (2002) showed cartilage degradation in collagen induced arthritis is delayed in iNOS knockout mice, suggesting that NO from iNOS is involved in joint pathology.

Clancy (1999) found that in bovine monolayer chondrocytes the production of NO led to decreased proteoglycan synthesis upon stimulation with fibronectin fragments. These studies suggest that NO produced by iNOS is degradative within these models leading to the progression of OA with inhibition protecting against OA progression. This suggests an antiinflammatory role for iNOS inhibitors in the treatment of OA.

Negative effect of iNOS inhibition

Several other studies support a protective role for iNOS production. McCartney-Francis et al (2001) used a rat streptococcal cell wall (SCW) induced arthritis to look at the effects of L-NMMA, L-NIL and AG. L-NMMA, a total NOS inhibitor, significantly reduced NO, synovial inflammation and tissue damage, supporting the previous findings. L-NIL and AG, however, led to the opposite effect suggesting that either iNOS derived NO has a protective role, or that the constitutive NOS isoforms may be involved in joint pathology. A murine AIA study (Veihelmann et al 2002) showed that, in the acute phase, treatment with L-NIL led to increases in swelling, leukocyte adhesion, leukocyte infiltration and expression of adhesion molecules. A previous study by the same group using iNOS KO mice showed exacerbation of joint inflammation with increased swelling and leukocyte infiltration.

Stefanovic-Racic et al (1997) showed that in rabbit cartilage shavings IL-1β led to decreased PG synthesis and increased degradation. NOS inhibition with L-NMA and thiocitrulline reversed the effects on PG synthesis, but increased degradation by collagenase, stromelysin and gelatinase (MMP) activation. The same group found similar results in bovine cartilage slices (Stefanocvic-Racic et al 1996) and primary human chondrocytes (Hauselmann et al 1998). This suggests that NO can have both protective and degradatory effects.

1.4 - Loading of articular cartilage

Mechanical loading is important in the regulation of articular cartilage structural integrity. This is shown in normal joints where load bearing areas are thicker, have higher PG content and are mechanically stronger, with the cells larger and containing more cellular organelles than non-loaded areas (Urban 1994). Reduced loading through immobilisation of one limb is deleterious to cartilage integrity, leading to tissue atrophy with reduced GAG content, elevated water content, reduced cartilage stiffness, reduced GAG synthesis and reduced aggregation of PGs (Behrens et al 1989). These effects are reversible following low levels of exercise, but the weakened cartilage is more susceptible to damage by severe exercise (Palmoski and Brandt 1981). Similarly, overloading caused by strenuous running exercise regimes reduce GAG content and produce local tissue damage at the contact sites of the cartilage (Palmoski and Brandt 1981) while moderate regimes enhance GAG content and cartilage thickness locally (Kiviranta et al 1988).

The loading of cartilage leads to a number of physicochemical changes including mechanical deformation, altered hydrostatic pressure, loss of water, streaming potentials, altered pH and osmolality that may all influence the metabolic activity of chondrocytes (Urban 1994). The tissue deforms under load as does the cell, leading to a rise in the hydrostatic pressure in the matrix within milliseconds. If the load is removed immediately, cartilage returns to its original conformation and the pressure falls. Load disturbs the osmotic equilibrium with prolonged loading causing the expression of fluid, increasing the concentration of PGs and cations in the matrix. Protein synthesis is very sensitive to intracellular ionic composition and rates fall with changes in extracellular fluid content. It is not surprising, therefore, that the extent of changes in the matrix vary with the loading pattern. The degree of deformation also depends on the integrity of the collagen meshwork. OA cartilage deforms more than normal cartilage under the same load and degenerate joints have a lower PG content and lose fluid much quicker than normal joints. Superficial cartilage is more compliant than deeper cartilage so the same load will have different effects on the cells from different zones and sites in the same joint (Urban 1994).

Other factors may be important. Oxygen levels may be altered by exercise and, although the cell metabolises mainly through anaerobic glycolysis, changes in oxygen tension have big effects on matrix synthesis and cell growth. Load induced solute movement can influence the rate at which growth factors and cytokines reach the cells and can alter cell metabolism. Static loading of cartilage decreases PG synthesis in a dose-dependent fashion (Gray et al 1989). The loss of water can lead to decreased pH and increased osmolality that may be responsible for the decreased PG synthesis. Pressures can reach 4-5MPa during walking and as high as 20MPa during some activities. Pressures of 5-50MPa may influence cell morphology, reduce exocytosis, dissociate cytoskeletal elements, reduce protein synthesis and inhibit membrane transport (Urban 1994). Pressures above 15-20MPa can lead to disorganisation of the golgi apparatus and microtubules; this cytoskeletal disaggregation may explain the decreased PG synthesis at high pressures (Hall et al 1991).

1.4.1 - Chondrocyte mechanotransduction (Figure 1.7)

Many cell types are exposed to mechanical stimuli within the body, however, the level and type of force experienced alters. Mechanical forces can influence cell signal transduction, gene expression, growth, differentiation and survival (Chen and Ingber 1999). Within cartilage it is possible that chondrocytes could be exposed to a variety of mechanical stimuli, including stretch, shear or compressive forces. Chondrocytes are also capable of generating a contractile force that can model the ECM, and this is mediated by the actin cytoskeleton (Zaleskas et al 2004). Chondrocytes require the ability to sense the mechanical signals within their environment and to transduce these signals into an appropriate response. This requires the presence of cell transmembrane receptors such as stretch activated ion channels, CD44 (hyaluronan receptor), anchorin II (collagen type II receptor) and integrin receptors (Loeser 2000).

Integrins have been implicated in a variety of chondrocyte cell functions including adhesion, proliferation, chondrogenesis and gene expression (Hering 1999) and the evidence suggesting integrins as mechanoreceptors is growing (Aplin et al 1998; Giancotti and Ruoslahti 1999; Wright et al 1997; Wright et al 1996). Integrins are heterodimeric transmembrane glycoproteins made up of an α and a β subunit (Hynes

1992). These contain a large extracellular domain that binds its ligand, a single transmembrane domain and a short cytoplasmic tail that interacts with intracellular signalling molecules and the actin cytoskeleton, enabling signal transduction (Shyy and Chien 1997). Divalent cation binding is essential for integrin receptor function. Both inside-out signalling, where a conformational change occurs between the active and inactive states, and outside-in signalling, where intracellular events are triggered, have been described (Hynes 1992). There are at least 16 α and 8 β subunits that combine to form more than 20 specific integrin receptors. The α5β1 integrin is required for membrane hyperpolarisation associated with 0.33Hz mechanical stimulation, and this suggests it has a role as a mechanoreceptor in chondrocytes (Wright et al 1997).

ECM binding causes clustering of integrins, with recruitment of adaptor proteins such as paxillin, talin and α-actinin linking the β integrin with the actin cytoskeleton (Howe and Juliano 1998; Hughes and Pfaff 1998). Recruitment of intracellular signalling molecules to the site leads to formation of a focal adhesion complex (FAC) (Aplin et al 1998; Shyy and Chien 1997). The tyrosine kinase focal adhesion kinase (FAK) is amongst the first molecules recruited to the FAC (Schwartz 2001). FAK autophosphorylation creates a binding site for the kinases Src and Fyn (Schaller et al 1994; Schlaepfer et al 1994). Both Src and FAK can phosphorylate other components of the FAC, including paxillin and tensin (Schlaepfer et al 1997; Vuori et al 1996). Tyrosine phosphorylation of FAK and paxillin, as well as β-catenin, is seen within 1 minute of 0.33Hz mechanical stimulation, a response that is lost by blocking the integrins (Lee HS et al 2000). Disruption of the actin cytoskeleton inhibits membrane hyperpolarisation, suggesting its importance (Wright et al 1996). β-catenin is associated with integrin signalling via the serine/threonine protein kinase, integrin-linked kinase (ILK) (Novak et al 1998). 0.33Hz mechanical stimulation also leads to the activation of the serine threonine kinase, PKC (Lee HS et al 2002), inhibition of which prevents membrane hyperpolarisation (Wright et al 1996). PKCα translocates to the cell membrane and associates with receptor for activated C-kinase (RACK-1), a regulatory protein, and β1 integrin following 30 seconds mechanical stimulation (Lee HS et al 2002). PKC is thought to have a role in the control of signalling through another serine/threonine kinase pathway, the MAPKs (Aplin et al 1998).

There is some evidence suggesting that MAPKs are activated by mechanical stimuli and these may be important in cellular responses to mechanical stimulation, including transcription factor activation, gene regulation, cell survival and cell differentiation (Aplin et al 1998; Giancotti and Ruoslahti 1999). Compression of cartilage explants has been shown to phosphorylate ERK 1 and 2, p38 MAPK and stress activated protein kinase (SAPK)/ERK (SEK-1) kinase of the JNK pathway in a time dependent manner (Fanning et al 2003). Studies in cell types other than chondrocytes have also shown MAPK activation due to mechanical stimulation, with ERK, p38 and JNK all being activated by mechanical strain in rat cardiac myocytes (MacKenna et al 1998).

Integrins may interact with ion channels, growth factor receptors and other transmembrane receptors, forming an integrated signalling complex (Ruwhof and van der Laarse 2000). β1 integrins colocalise with Na^+-K^+ ATPase, epithelial sodium channels and voltage activated calcium channels in mechanoreceptor complexes in mouse limb bud chondrocytes (Shakibaei and Mobasheri 2003). This may allow cellular responses and signalling cascades to be regulated more effectively. Stretch activated ion channels (SAC) and the glutamate receptor NMDA are involved in the membrane hyperpolarisation and tyrosine phosphorylation associated with 0.33Hz mechanical stimulation (Salter et al 2004; Wright et al 1996). Influx of calcium through SAC may allow direct activation of calcium dependent pathways and is seen following mechanical stimulation (Roberts et al 2001). ATP release and increased intracellular calcium levels are seen within 500 milliseconds of mechanical stimulation (Graff and Lee 2003). The calcium wave activates phospholipase C (PLC) that produces inositol triphosphate (IP3) and this second messenger can travel via gap junctions to adjacent cells, stimulating further calcium wave production. (D'Andrea et al 2000). ATP has been identified as a molecule involved in the membrane hyperpolarisation of normal monolayer chondrocytes following 0.33Hz MS for 20 minutes. ATP is upstream of IL-4 in the signal transduction leading to activation of apamin sensitive small conductance calcium activated potassium channels (Millward-Sadler et al 2004).

The membrane hyperpolarisation may contribute to the negative regulation of apoptosis (Gilbert et al 1996; Wang et al 1999). The activation of proteases by large conductance calcium activated potassium channels in the phagocytic vacuole of

leukocytes occurs through a combination of membrane depolarisation and increased calcium concentration (Ahluwalia et al 2004). Mechanical stimulation at a frequency of 0.33Hz causes membrane depolarisation in OA chondrocytes (Millward-Sadler et al 2000a), and may be involved in activating intracellular molecules in a similar fashion to the activation of proteases seen in the leukocyte vacuole.

The production of soluble mediators such as cytokines and growth factors in response to mechanical forces has been described in mechanosensitive cells including osteoblasts (Salter et al 1997). *In vitro* mechanical stimulation of chondrocytes shows similar autocrine/paracrine signalling (Millward-Sadler et al 1999). As chondrocytes are the only cells present in cartilage they must function as both sensor and effector cells. However other cell types in the joint may also be affected (Millward-Sadler et al 2003). The antiinflammatory cytokine IL-4, and neuropeptide substance P, are secreted early in the mechanotransduction cascade, with production seen within 20 minutes suggesting release from preformed stores. Substance P has been shown to be upstream of IL-4 in the signal cascade, acting through the NK-1 receptor to induce IL-4 release. IL-4 and substance P appear to be necessary, but not sufficient, for gene expression changes induced by MS, suggesting cross-talk may occur with other integrin dependent signalling pathways activated by MS (Millward-Sadler et al 2003). IL-4 is suggested to block the activation of latent proMMPs rather than their production (van Lent et al 2002) and can protect synovial cells from NO-mediated apoptosis (Relic et al 2001). IL-4 was shown to suppress the IL-1β mediated increase in MMP3 mRNA, protein and enzyme activity in human articular chondrocytes (Nemoto et al 1997). IL-4 has been suggested to act by suppressing IL-1gene expression (Wong et al 1993) and increasing the synthesis of IL-1ra via STAT6 (Ohmori et al 1996). However, Palmer et al (2002) suggests that IL-4 did not activate IL-1ra in chondrocytes, and that IL-1 induced IL-1ra took 24 hours to reach detectable levels. IL-4 may also increase the inactive IL-1 receptor, IL-1RII, another potential mechanism by which it could negatively regulate IL-1 signalling (Colotta et al 1993).

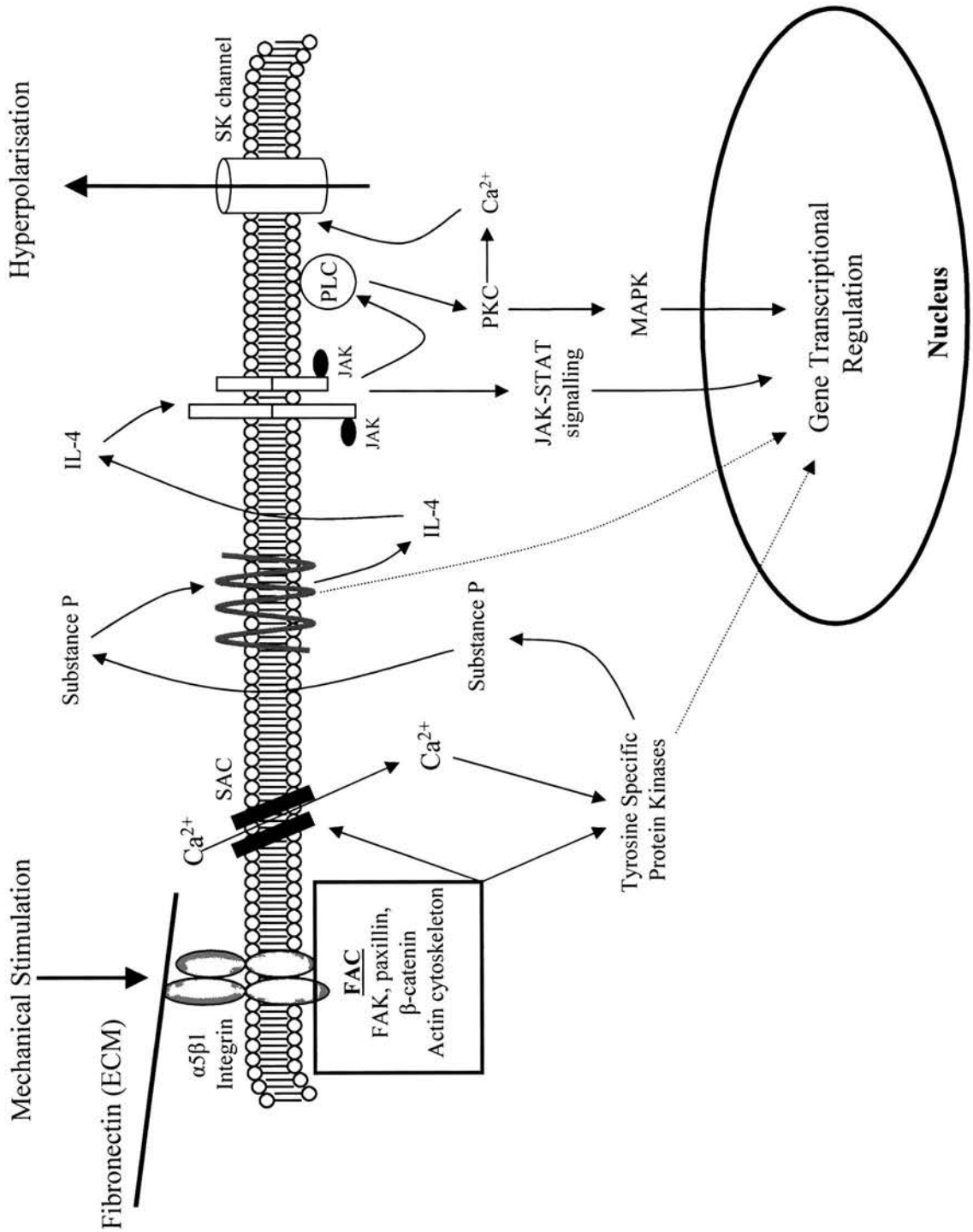


Figure 1.7 – Normal chondrocyte mechanotransduction pathway. Mechanical signals are sensed through attachment to the ECM via the integrins. The function of integrins can be modulated by associated proteins such as CD47. Stretch activated ion channels also influence the activation of intracellular molecules. The FAC associated with the integrins intracellular domain contains molecules that initiate intracellular signalling. The autocrine/paracrine release of substance P and IL-4 cause activation of PLC and PKC releasing calcium and activating sodium activated potassium channels that causes membrane hyperpolarisation. The release of ATP and glutamate have also been implicated in membrane hyperpolarisation, although there exact position in this pathway is not known.

1.4.2 - OA chondrocyte mechanotransduction (Figure 1.8)

OA cartilage derived chondrocytes show different responses to 0.33Hz stimulation in monolayer compared to cells from normal joints. Membrane depolarisation is seen in place of the hyperpolarisation in normal cells, with an absence of the rise of aggrecan mRNA (Millward-Sadler et al 2000a; Millward-Sadler et al 2000b). The depolarisation response involves tetrodotoxin sensitive sodium channels rather than activation of SK channels, which are also present on OA chondrocytes. The loss of the aggrecan response would be expected to have significant effects on cartilage structure and function. The effects of the changes in the electrophysiological response are less clear as it is not involved in the upregulation of aggrecan in normal cells (Millward-Sadler et al 2000a). Nevertheless it suggests fundamental differences in mechanotransduction. The altered ECM seen in OA, as discussed earlier, will change the response to the mechanical stimuli sensed by the cells, and adaptation to the altered environment may require a different threshold of stimulus before cellular activation events are stimulated. Altered expression of integrins in OA (Ostergaard et al 1998) will also influence the mechanotransduction process, although the depolarisation response associated with OA monolayer chondrocytes at 0.33Hz appears to be via the same integrin as the hyperpolarisation response seen in normal chondrocytes, $\alpha 5\beta 1$ (Millward-Sadler et al 2000a). Cytoskeletal disruption did not affect depolarisation which suggests it is unnecessary for the response. An NMDA antagonist had no effect on depolarisation of OA chondrocytes, so alteration of NMDA receptor signalling in OA may be critical in the abnormal response to MS (Salter et al 2004).

FAK, paxillin and β-catenin show prolonged tyrosine phosphorylation after mechanical stimulation in OA cells (Lee HS et al 2000), and this may be due to changes in the phosphatase activity known to regulate these molecules (Giancotti and Ruoslahti 1999). Mechanical stimulation at 0.33Hz in OA also induces PI3kinase (Millward-Sadler et al 2000a) that is regulated via integrins, through integrin-linked kinases that are within the FAC (Dedhar 2000). Changes in the activation of the kinases PKC and PI3K may adversely influence proteoglycan metabolism (Arner and Pratta 1991; Kolanus and Seed 1997).

Autocrine IL-1β production is seen in addition to IL-4 and substance P after MS in OA chondrocytes, which is likely to have catabolic effects as IL-1β can induce MMP release and can influence cartilage ECM regulation (Millward-Sadler et al 2000a). A variable response of hyper- and de-polarisation is seen upon addition of IL-4 to chondrocytes. IL-4 can signal via its type II receptor (Il-4Ralpha/IL-13R), causing tyrosine phosphorylation of JAK1 and TYK2 and signalling via STAT6. IL-4 signalling can also occur through the Type I receptor (IL-4Ralpha/common gamma chain) activating JAK1 and JAK 3 and can stimulate STAT5 activity. It may be through activation of these different receptors that IL-4 is involved in both the hyperpolarisation seen in normal chondrocytes and the depolarisation in OA chondrocytes (Salter et al 2002). SOCS-1 binds and inhibits the kinase activity of JAKs, inhibiting IL-4 mediated JAK1 and STAT6 activation (Losman et al 1999). SOCS-3 mediates IL-1β inhibition of STAT5 activity (Boisclair et al 2000). These factors could be produced in OA cells so altering the response due to IL-4 stimulation.

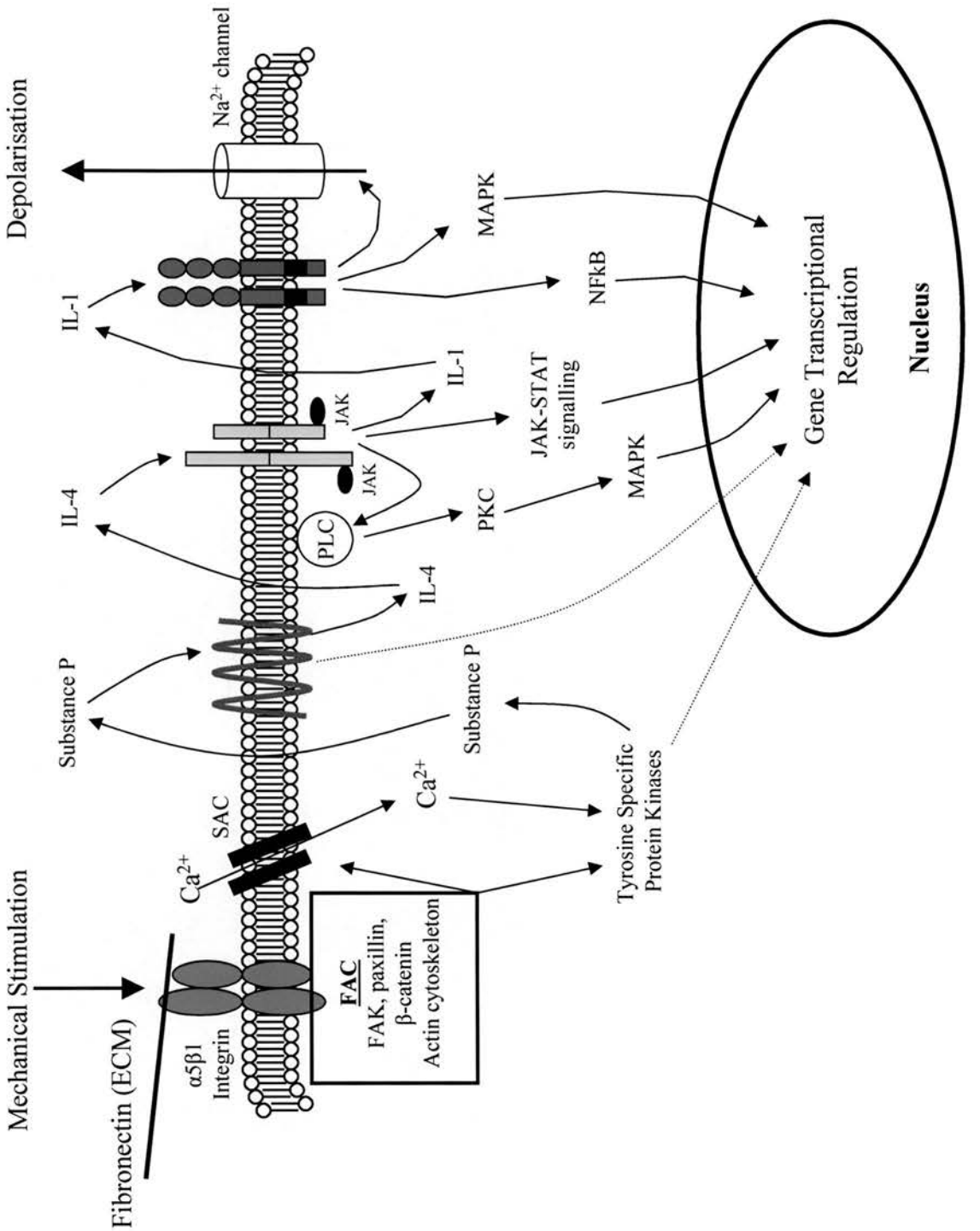


Figure 1.8 – OA chondrocyte mechanotransduction pathway. Mechanical signals are sensed through attachment to the ECM via the integrins. The function of integrins can be modulated by associated proteins such as CD47. Stretch activated ion channels also influence the activation of intracellular molecules. The FAC associated with the integrins intracellular domain contains molecules that initiate intracellular signalling. The autocrine/paracrine release of substance P, IL-4 and IL-1 cause activation of PLC and PKC, releasing calcium and activating sodium channels that cause membrane depolarisation. The release of ATP and glutamate are not involved in membrane depolarisation, although their receptors are present.

1.4.3 - Other studies of chondrocyte mechanotransduction

A number of different types of apparatus have been designed to apply strains of different frequencies, magnitudes and types to cells in monolayer culture, in three dimensional (3-d) culture and in explants (Reviewed in Brown 2000). The system used to apply strain is important as alterations in the applied strain will occur between systems. It is important to highlight some of the other mechanisms that have been used to study mechanotransduction in chondrocytes, with the relative merits of these systems compared to that of our own.

Firstly, our system uses chondrocytes seeded in monolayer culture with mechanical strain transmitted to the cells through the stretch of the culture surface. It has been reported that chondrocytes dedifferentiate in monolayer culture (Mahmood et al 2004), however our cells have been shown to express markers for differentiation at the time of experimentation (Edinburgh University OA research group unpublished observations). The fact that the cells are in monolayer, however, means that they do not have a fully formed matrix as would be seen *in vivo*. This will result in alterations in the level and types of strain experienced by the cell as well as increased access to the cells for signalling molecules such as cytokines and growth factors. Provided these limitations are understood, however, this method remains a very useful one in the study of chondrocyte mechanotransduction. This is due to the relative ease with which mRNA, protein and enzyme activity studies can be performed and the speed at which cellular reactions can be stopped and analysed at the end of each experimental protocol.

The application of mechanical strain in our system appears to be sensed by the cells through the $\alpha 5\beta 1$ integrin, however it is unlikely that this is the only receptor through which strain is being sensed. The use of an antibody specific for certain cell surface molecules with an attached metal fragment has been used (Wang and Ingber 1995). Here the application of a magnetic field can stimulate the cells via the specific receptors the antibody is bound to giving data about the differences between mechanotransduction through certain cell surface molecules. Care must be taken, however, as the application of a magnetic field may itself have effects on cellular processes.

Several other groups have applied cyclic tensile strain through stretching or flexing the culture membrane. Perhaps the most relevant of these is Agarwal et al (2004). Here, the application of strain to monolayer chondrocytes was shown to influence activity of the transcription factor NFκB dependent upon the magnitude of the applied strain.

D'Andrea et al (2000) used the stimulation of single chondrocytes using a micropipette to show alterations in calcium concentration within the cell. This was followed by showing that mechanical strain causes the activation of phospholipase C, which in turn stimulates Inositol 1,4,5-triphosphate (IP3) release that is responsible for alterations in calcium concentrations. It was suggested that the IP3 may actually leave the stimulated cell, travel to an adjacent cell and stimulate calcium signalling.

Methods for the application of strain in monolayer other than through the stretching of culture membranes include fluid flow and hydrostatic pressure. Hung et al (2000) used a laminar flow chamber to exert strain on cultured monolayer chondrocytes. Activation of ERK1/2 was seen following 5-15 minutes of fluid flow with pharmacological inhibitors used to show that this response was not dependent upon calcium mobilisation. This technique allows the accurate application of shear stress to the cells, however the release of signalling molecules that may be involved in mechanotransduction into the medium may not exert such an effect on the cells due to the flow of the fluid. The use of a cone viscometer has also been employed to exert fluid induced shear stress on monolayer chondrocytes (Smith et al 1995). This study identified increased PGE_2 and TIMP levels following the shear stress associated with fluid flow. The direction of the fluid flow is the main draw back here, with cells aligning to the flow, which may affect the way in which they sense the strain. A novel mechanism to investigate the effect of fluid flow on chondrocytes was employed by Graff and Lee (2003). Here the use of a plate reader that injects medium into wells containing cells leads to increased ATP release and calcium mobilisation, which were monitored immediately using the same plate reader. The problem with this approach is that the strain is very hard to measure and keep uniform.

Smith et al (1996 and 2000) investigated the effects of the application of hydrostatic pressure on chondrocytes, identifying the role this plays on matrix synthesis. Here the culture plates were placed in medium filled bags and immersed in a water tank, the compression of which was used to apply the pressure. The role of strain on cartilage matrix homeostasis will be discussed in more detail later. Browning et al (2004) also investigated the application of hydrostatic pressure on monolayer chondrocytes, showing effects on calcium concentrations.

Mechanical stimulation using cells in 3-d matrix more closely resembles the *in vivo* environment. In this situation the cells retain the chondrocytic phenotype in long term culture (Yoon et al 2002). They are more likely to experience strains that are similar to those seen *in vivo* due to similar attachments to the ECM and production of a pericellular matrix. This can be achieved by culture in agarose or alginate beads. Chowdhury et al (2004) used agarose constructs to culture chondrocytes in a 3-d matrix. The constructs were subjected to compressive stress with the study identifying the $\alpha5\beta1$ integrin as an essential molecule in compression-induced changes in cellular metabolism. Roberts et al (2001) used the same system to show calcium concentration alterations due to mechanical compression. The difficulty with this system is seen during the measurement of transient changes such as that seen during cell signalling. The phosphorylation of proteins is very often the mechanism through which they are activated and the isolation of these proteins immediately at the end of the experimental protocol is complicated by the presence of the matrix itself. There are also problems associated with the uniform application of strain to the cells within the construct. Wu et al (2001) cultured chondrocytes in a novel 3-d matrix composed of collagen, with mechanical stimulation applied by stretching this matrix. The study showed that mechanical stimulation induced a factor known to control chondrocyte proliferation and differentiation, Indian hedgehog.

Finally, the use of intact cartilage allows cells to be studied within their natural matrix. Within this system they should sense strains in the way they do *in vivo*. Compression of cartilage explants has been used in several studies. Fanning et al (2003) used cartilage explants to show the sequence of phosphorylation of MAPKs due to mechanical compression. Lucchinetti et al (2004) also used cartilage explants and compression to show that levels of $\alpha5$ integrin increased due to strain while $\beta1$

integrin remained constant. The difficulty with this system is in understanding the exact strains that are being applied to the cells, and which of these are causing any effect seen. There are also problems in monitoring the transient changes during cell signalling which are even more difficult due to the cartilage matrix than seen using agarose and alginate culture.

Other mechanisms include the use of animal models of arthritis. These allow the effects of certain mechanical loads to be evaluated in vivo, allowing identification of global effects on the joint. However, the identification of the exact mechanisms through which changes occur is very difficult.

Due to the difficulties associated with monitoring the transient effects of mechanical stimulation on cellular signalling molecules, it is more common for effects of strain to be determined by detection of more stable molecules such as matrix molecules, or the production of molecules such as NO. The effects of strain on these molecules will be discussed in more detail in the next section.

1.5 - Mechanical and cytokine stimulation studies looking at NO

The problem with the study of mechanotransduction is that cartilage is a heterogeneous tissue. The cells will therefore experience different strains and have different responses to mechanical stimuli depending upon their position within the cartilage (Langelier et al 2000). The magnitude of strain experienced within the joint and the stresses and strains that are experienced by chondrocytes *in vivo* are not known. The only study to measure the levels of strain experienced used a sensor implanted into an artificial hip joint to calculate physiological levels (Hodge et al 1986). The findings reveal high local pressures of up to 18MPa that are not uniform across the joint.

There have been only a limited number of studies that have investigated the interactions between mechanical stimulation and cytokine stimulation in chondrocytes where iNOS and nitrite were used as measures of response (summarised in Table 1.3). Initial experiments involving strain looked at its effects on proliferation and proteoglycan synthesis, these experiments will be discussed in more detail in Section 1.6.

A study by Lee DA et al (1998) showed strain (15% compressive strain at 0.3, 1 and 3Hz for 48 hours) having an effect on LPS induced nitrite production in bovine articular chondrocytes seeded in agarose. This group have since used the same strain regimen (15% strain, 1Hz for 48 hours) to show that IL-1β stimulates nitrite and PGE_2 that are inhibited by strain (Chowdhury et al 2001)
Lapine articular chondrocytes subjected to IL-1β were shown to produce iNOS mRNA, protein and nitrite. This was inhibited by the application of cyclic tensile strain at 20% stretch and 0.05Hz (Gassner et al 1999) and showed basal nitrite levels were not affected by strain (Gassner et al 2000). Xu et al (2000) used the same strain system but 6% stretch at 0.05Hz to show a similar reversal of IL-1β induced iNOS mRNA and nitrite as well as COXII mRNA and PGE_2. Interestingly this group studied the effects of adding IL-1β prior to the application of strain and found that after 1 hour the effects of strain were halved, and after 2 hours strain had no effect on iNOS, COXII, nitrite and PGE_2. Rabbit temperomandibular junction chondrocytes have been used by this group to investigate the effects of different magnitudes of

strain, with the use of a flexercell strain unit to apply cyclic strain at 10Hz, 20% elongation and showing the reversal of IL-1β induced iNOS and COXII mRNA as well as nitrite and PGE_2 in these cells (Agarwal et al 2001). Long et al (2001) showed that rabbit articular chondrocytes respond similarly to cyclic tensile strain, reducing the TNFα-induced iNOS and COXII mRNA levels.

Porcine chondrocytes appear not to follow the same trend as the other species discussed so far. Fermor et al (2001 and 2002) applied static and cyclic compression over a range of magnitudes to porcine articular cartilage explants and found that strain upregulated nitrite and PGE_2 production. This suggests that species differences exist in the regulation of iNOS production and the response of chondrocytes to mechanical stimulation. This should be considered before drawing conclusions from the results obtained from different species.

Author	Cell Type	Mechanical stimulus	Other Stimuli	Response
Agarwal 2001	Lapine TMJ monolayer chondrocytes	Flexercell 10Hz 20% elongation rate CTS	1ng/ml IL-1β	Strain → ↓iNOS, COXII and MMP1 mRNA as well as nitrite and PGE2. ↑TIMP-2 Prevented IL-1β inhibition of PG synthesis
Xu 2000	Lapine articular chondrocytes in monolayer	CTS 0.05Hz 6% stress	IL-1β 1ng/ml	CTS reverses IL-1 induced iNOS and COX II mRNA, nitrite and PGE2. CTS also increases TIMP and reverses IL-1β reduced coll II mRNA. CTS and IL-1β in combination increase aggrecan mRNA. CTS and IL-1 have to be simultaneous – pre CTS has no effect on IL-1β induced nitrite and iNOS mRNA – pre IL-1β decreases effect of CTS.
Gassner 2000	Lapine articular chondrocytes	CTS for 24hr	IL-1β	CTS doesn't affect basal NO CTS decreases IL-1β induced NO
Gassner 1999	Lapine shoulder and knee monolayer chondrocytes	CTS = 0.05Hz 20% elongation	IL-1β dose response	iNOS mRNA, protein and nitrite detected due to IL-1β and inhibited by CTS PG synthesis also restored
Chowdhury 2001	Bovine chondrocytes in agarose	15% strain, 0.3, 1, 3Hz, 48 hours	IL-1β conc and L-NIO conc	Dose increase in nitrite and PGE2 due to IL-1. Strain inhibits nitrite and PGE2, reversed by L-NIO suggesting NO involved in PGE2 synthesis. Proliferation inhibite by IL-1 and reversed by L-NIO so NO involved. PG synthesis not affected by NO.
Lee DA 1998	Bovine chondrocytes in agarose	15% strain, 0.3, 1, 3Hz, 48 hours	LPS, L-NAME, Dexamethasone	LPS→↑nitrite. Strain, L-NAME and DEX →↓ nitrite L-NAME partially blocked nitrite due to strain and LPS, and reversed strain related increase in proliferation. PG synthesis is unaffected by NO
Fermor 2002	Porcine cartilage explants	Intermittant compression range		Strain increased NO and PGE2. PGE2 was dependent upon NO.
Fermor 2001	Porcine cartilage explants	Static and cyclic (0.5Hz) stain at 0.1, 0.5 and 1MPa	1400W	Some of the strain regimen increase nitrite and iNOS protein, but cNOS is not detected. 1400W reduces NO
Long 2001	Rabbit articular chondrocytes	CTS	TNFα	TNFalpha induced iNOS, COXII mRNA. CTS inhibits this.
Long 2002	Osteoblast-like periodontal ligament cells	Equibiaxial tensile strain	IL-1β	Strain inhibits COXII and PGE2 production due to IL-1β

Table 1.3 – Mechanically and cytokine stimulated chondrocyte experiments measuring iNOS and NO levels. CTS=Cyclic tensile strain.

1.6 - PG synthesis following mechanical stimulation and involvement of NO

There have been a number of studies looking at the effects of different mechanical loads on chondrocytes and cartilage, with proteoglycan synthesis being one of the parameters monitored. Cartilage explant, three-dimensional cultures and monolayer cultures of chondrocytes have all shown that, following cyclic mechanical stimulation, there is an increase in proteoglycan production, while static compression leads to decreased proteoglycan production (Lee DA et al 2000; Sauerland et al 2003; Valhmu et al 1998; Waldman et al 2003). Loading frequency and maximal stress are also important factors in the outcome, with shear, stretch and compression all able to induce anabolic responses (Wong et al 1999).

The possible involvement of NO in the altered proteoglycan synthesis due to mechanical stimulation has been looked at in several studies listed in Table 1.4.

Lee DA and Bader (1997) looked at proteoglycan levels following 48 hours of 15% compressive strain of bovine articular chondrocytes seeded in agarose constructs. Proteoglycan levels were increased by 50% at 1Hz, while 0.3Hz and static strain decreased levels by 5-15%. The same group then looked at the effects of NO on PG synthesis (Lee DA et al 1998). Strain increased PG synthesis and decreased nitrite levels associated with LPS stimulation, with NOS inhibitors showing that strain increased PG synthesis is only slightly influenced by NO. Chowdhury et al (2001) then looked at IL-1β in the same system, showing it decreased proteoglycan synthesis in both an NO dependent and independent manner.
Primary rabbit articular chondrocytes seeded in monolayer were subjected to cyclic tensile strain (0.05Hz 20% elongation for up to 96 hours) and IL-1β stimulation (Gassner et al 1999). IL-1β caused decreased proteoglycan synthesis and increased iNOS mRNA, protein and nitrite. Strain slightly reduced PG synthesis when applied alone, however it restores PG synthesis levels following IL-1β stimulation as well as reducing iNOS mRNA, protein and nitrite levels. Xu et al (2000) showed that IL-1β increases iNOS mRNA and nitrite and the application of strain reverses these. IL-1β alone decreased proteoglycan synthesis significantly, with strain reversing this effect.

Gassner et al (2000) showed the involvement of NO, with L-NMA partially restoring the inhibition of PG synthesis associated with IL-1β stimulation.

Several other studies have shown similar effects of strain on TNFα mediated PG suppression (Long et al 2001), L-NMA inhibited NO production and increased PG synthesis while SNAP increased NO and decreased PG (Liu et al 2001) and NO mediated the reduced PG synthesis associated with IL-1β in equine articular cartilage, shown using the inhibitor L-NIO, and donor DETA-NONOate (Bird et al 2000).

Author	Cell Type	Mechanical stimulus	Other Stimuli	Response
Agarwal 2001	Rabbit TMJ monolayer C0	Flexercell 10Hz 20% elongation rate CTS.	1ng/ml IL-1β	CTS→↓iNOS mRNA and nitrite CTS→Prevented IL-1 inhibition of PG synthesis
Xu 2000	Rabbit articular chondrocytes in monolayer	CTS 0.05Hz 6% stress	IL-1β 1ng/ml	CTS reverses IL-1 induced iNOS mRNA and nitrite. CTS and IL-1 in combination increase aggrecan mRNA
Gassner 2000	Lapine articular chondrocytes	CTS for 24hr	IL-1β L-NMA	CTS→Reversed ↑NO and ↓PG due to IL-1 L-NMA→↓NO and partially restored PG due to IL-1
Gassner 1999	Lapine articular monolayer chondrocytes	CTS = 0.05Hz 20% elongation	IL-1β dose response	IL-1→ ↑iNOS mRNA, protein and nitrite and decreased PG synthesis CTS→ Inhibits iNOS mRNA, protein and nitrite and reverses PG synthesis due to IL-1
Chowdhury 2001	Bovine chondrocytes in agarose	15% strain, 0.3, 1, 3Hz, 48 hours	IL-1β conc and L-NIO conc	IL-1→↑nitrite Strain→↓ nitrite due to IL-1 PG synthesis is increased by strain and this increase is inhibited by IL-1 and further inhibited by L-NIO, so NO involved in increased PG due to strain.
Lee DA 1998	Bovine chondrocytes in agarose	15% strain, 0.3, 1, 3Hz, 48 hours	LPS (10ug/ml), L-NAME, Dexamethasone	LPS→↑ nitrite Strain→↓ nitrite due to LPS L-NAME partially blocked nitrite due to strain plus LPS. PG synthesis is only slightly influenced by NO
Lee DA 1997	Bovine chondrocytes in agarose	0.1, 1, 3Hz 15% compressive strain 48hrs		↑PG synthesis at 1Hz by 50% ↓PG synthesis at 0.3Hz and static by 5-15%
Lee MS 2003	Monolayer human chondrocytes	Intermittent hydrostatic pressure (10MPa, 1Hz, 4hr)	LPS (1ug/ml)	LPS→ ↑iNOS mRNA and nitrite and ↓aggrecan mRNA Strain→ ↓iNOS and nitrite and partially reversed ↓aggrecan mRNA caused by LPS
Long 2001a	Articular chondrocytes	CTS	TNFα	TNFα induced iNOS mRNA. CTS inhibits this.
Liu 2001	Human lumbar disc cubes	Hydrostatic pressure in syringe (3atm and 30atm)	L-NMA	3atm→ ↑PG synthesis 30atm→ ↓PG synthesis L-NMA → increased PG due to 3atm and relieved decreased PG due to 30atm
Bird 2000	Equine articular chondrocytes		IL-1β L-NIO DETA-NONOate	NO fully mediated the decrease PG synthesis due to IL-1

Table 1.4 – Mechanically and cytokine stimulated chondrocyte experiments measuring proteoglycan synthesis - involvement of NO. CTS=Cyclic tensile strain.

1.7 – Aims and Objectives

The aim of this study was to investigate interactions between mechanical signalling, nitric oxide synthesis and PG synthesis in human chondrocytes. In order to achieve these aims a number of objectives were identified:

(I) Investigate NOS isoform expression in chondrocyte cell lines and in primary human chondrocytes

(II) Investigate the effects of mechanical stimulation on NOS expression

(III) Investigate the effects of mechanical stimulation and NO on PG synthesis

Chapter 2 - Materials and Methods

2.1 Human Articular Chondrocyte (HAC) culture

2.1.1 Isolation of HAC

Human articular cartilage was collected at surgery with informed consent from patients undergoing joint replacement. The cartilage surface was macroscopically graded according to the Collins and McElligott scoring system (Collins and McElligott 1960) (**Table 2.1**) and sections removed to be snap frozen and stored at -70°C for microscopic grading (Mankin et al 1971) (**Table 2.2**). Sterile forceps and scalpel were used to remove the cartilage from the bone surface, pooling cartilage of similar grading from the same joint surface within a single sample. Bone tissue was then soaked overnight in Formalin before being bagged and sent for incineration. Cartilage was placed in a tissue culture dish (NUNC) containing an antimicrobial solution composed of 10mM glutamine, 50 I.U/ml penicillin, 50μg/ml streptomycin and 12.5μg/ml amphotericin B (all Gibco, Paisley, UK), chopped into ~1mm^2 pieces and left at room temperature for 60-90 minutes. Antimicrobial solution was removed and fragments were then washed with sterile phosphate buffered saline (PBS) twice before incubation with 0.25% trypsin (Gibco) for 30 minutes at 37°C. Trypsin was then removed and the fragments again washed twice with PBS before addition of 3mg/ml collagenase (Sigma Poole, UK) in Iscoves Modified Dulbecco's Medium (MEM) (Sigma, Poole, UK) supplemented with 10% foetal calf serum (FCS) (First Link UK Ltd), 10 I.U/ml penicillin, 10μg/ml streptomycin (Gibco) and 2mM L-glutamine (Gibco) (complete medium) and left overnight in an incubator at 37°C in an atmosphere of 95%O_2, 5%CO_2. The following day the tissue culture dish was removed from the incubator and the sample was gently agitated using a sterile pastette before being passed through a sterile metal filter into a sterile centrifuge tube. The sample was then spun at 300g (1000rpm) in a MSE Mistral 2000R centrifuge (Sanyo-Gallenkamp) for 10 minutes. Supernatant was removed, the pellet resuspended in PBS and centrifuged under the same conditions. This wash step was repeated once more before the cells were resuspended in complete medium supplemented with 6.25μg/ml amphotericin B (Gibco) (seeding medium) and passed through a 70-micron filter (Becton Dickinson, Oxford, UK) into a new sterile centrifuge tube, removing any large cell clumps or pieces of debris.

Table 2.1 - Collins-McElligott scoring system (Collins and McElligott 1960)

Collins Grade	Criteria
0	No cartilage degeneration
I	Limited patches of fibrillation or softening in central area of articular cartilage No area of whole cartilage thickness loss No recognisable marginal osteophytosis No detectable synovial inflammation
II	Large area of fibrillation and fissuring without denuding bone Early marginal chondro-osteophytosis
III	At least one area (30% or less) of whole thickness cartilage loss and extensive fibrillation, fissuring and bone exposure More generalised synovial disease and obvious marginal osteophytosis
IV	Extensive cartilage loss and bone exposure Eburnation and bone grooving, destruction of intraarticular ligaments and fibrosis or atrophy of synovial fringes

Table 2.2 - Mankin histological/histochemical grading system for osteoarthritic cartilage (Mankin et al 1971)

Category	Subcategory	Score
Structure		
	Normal	0
	Surface irregularities	1
	Pannus and surface irregularities	2
	Clefts to middle zone	3
	Clefts to deep zone	4
	Clefts to calcified zone	5
	Complete disorganisation	6
Cells		
	Normal	0
	Diffuse hypercellularity	1
	Cloning	2
	Hypocellularity	3
Safranin O staining		
	Normal	0
	Slight reduction	1
	Moderate reduction	2
	Severe reduction	3
	No dye noted	4
Tidemark integrity		
	Intact	0
	Crossed by blood vessels	1
Total		**0-14**

2.1.2 Plating of Cells for Experiments

The cell number in the suspension was assessed using a Neubauer haemocytometer (**Figure 2.1**). With a coverslip in place, 10µl cell suspension was mixed with 10µl 0.4% Trypan blue (Sigma). 10µl of this was added to one chamber by touching the tip to the edge of the coverslip and allowing capillary action to fill the haemocytometer chamber. Cell number was then calculated by counting all the cells in the 1mm corner squares (**Figure 2.1**) with each 1mm square representing a total volume of $10^{-4}cm^3$. Therefore, as $1cm^3$ is equal to 1ml, the number of cells per ml is equal to the average count per 1mm square x 10^4.

Cells were seeded in 58mm Petri dishes (Nunc) containing 5ml total volume at $5x10^4$/ml in seeding medium. After seeding cells were incubated for 48-72 hours at 37°C to allow cells to adhere to the base of the dish. Cells were grown in monolayer for 7-10 days with complete medium added every 48-72 hours. When the dishes reached 80-90% confluence the medium was removed and replaced with complete medium without FCS added (serum free medium) for ~16 hours before commencing experiments. Each condition and time-point was analysed using a single culture dish without a replicate.

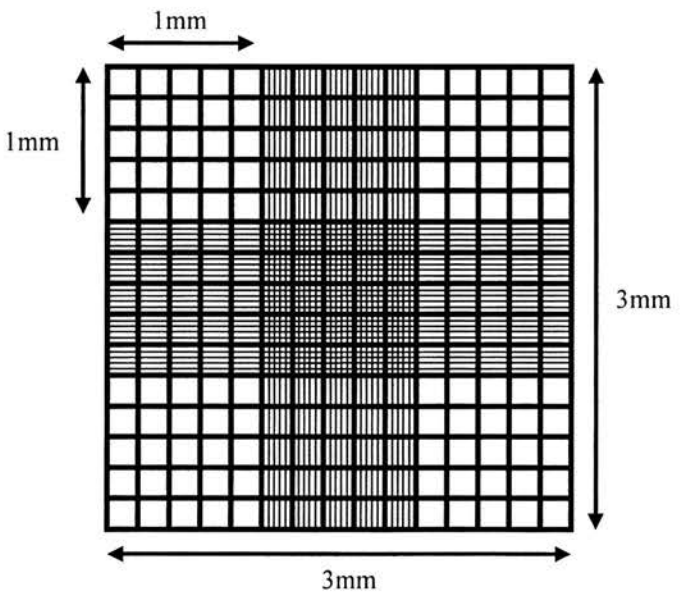


Figure 2.1 – Diagrammatic representation of Neubauer haemocytometer

2.1.3 iNOS Inhibitor experiments

iNOS inhibitor experiments were carried out using RPMI 1640 medium with no added arginine (Sigma). Primary OA HAC were grown to confluence as previously described and then serum starved for ~16 hours using the arginine free RPMI 1640 supplemented with 10 I.U/ml penicillin, 10μg/ml streptomycin (Gibco), 2mM L-glutamine (Gibco) and 100μM L-arginine (Sigma).

2.2 Culture of other cells

2.2.1 Chondrocyte Cell Line Culture

Transformed human chondrocyte cell lines C20A4 and C28I2 (Donated by Dr M Goldring) were grown at 37°C in an atmosphere of 95%O_2, 5%CO_2. Cells were cultured in complete medium and passaged every 6 days. Trypsin/EDTA solution (Gibco) was used to detach cells from the base of the tissue culture flask and cells were then washed in PBS and spun at 300g (1000rpm) for 10 minutes. Cells were resuspended in complete medium before being counted and seeded in the same way as described for primary HAC. Transformed cells reached confluence in a shorter time than primary HAC (3-4 days) and were then incubated in serum free medium for ~16 hours before experiments were carried out using a single culture dish without a replicate for each condition tested.

2.2.2 Culture of cells used as western blot positive controls

DLD-1

The human colorectal adenocarcinoma cell line DLD-1 was cultured as detailed above for the chondrocyte cell lines. These cells are known to produce iNOS in response to pro-inflammatory cytokine stimulation (Linn et al 1997; Salzman et al 1996). Upon reaching confluence these cells were stimulated with the cytokine cocktail containing IL-1β, TNFα, IL-6 and IFNγ (as detailed in Chemicals Reagents **Section 2.4**) for 16 hours before lysis and protein extraction as detailed in **Section 2.8** and used as a positive control for iNOS western blotting (Tinker et al 2003).

EAhy926

The human umbilical vein endothelial cell line EAhy926 (Donated by Dr S Brown) (Edgell et al 1983) was cultured as detailed above for the chondrocyte cell lines. Upon reaching confluence these cells were lysed and protein extracted as detailed in **Section 2.8** and used as a positive control for eNOS (Thum et al 2003).

2.3 Mechanical Stimulation

The mechanical stimulation apparatus used is a modification of that previously described (Wright et al 1992; Wright et al 1996) (**Figure 2.2**). Flexible 58mm tissue culture dishes (Nunc) were placed on rubber 'O' rings in polypropylene stimulation chambers. The lid was screwed down to hold the dish in position with the 'O' ring ensuring an airtight seal between the chambers above and below the dish (**Figure 2.3**).

Inlet ports on the stimulation chambers allow the entry of Nitrogen gas into the space below the dish, increasing the pressure in the lower chamber and causing the dish to balloon upwards, thus applying strain to both the base of the dish and to adherent HAC. Outlet ports then allow the release of the Nitrogen gas causing the pressures to return to normal and the dish returns to its resting state (**Figure 2.4**). Dr Malcolm Wright and Mr Paul Parker extensively calibrated the system by the use of strain gauges attached to the base of the culture dish to measure the strain induced by various pressures (**Figure 2.5**).

The frequency with which the dishes were exposed to strain is dictated by an electronic timer, with 0.33Hz (2sec on, 1 sec off) used in all experiments in this study. A gas pressure of 0.25atmospheres (190mmHg) was used which results in ~31,000 μstain on the bottom of the dish. Experiments were carried out at 37°C in an atmosphere of 95%O_2, 5%CO_2 using a single culture dish without a replicate for each condition tested.

Side Profile

Birds-eye-view

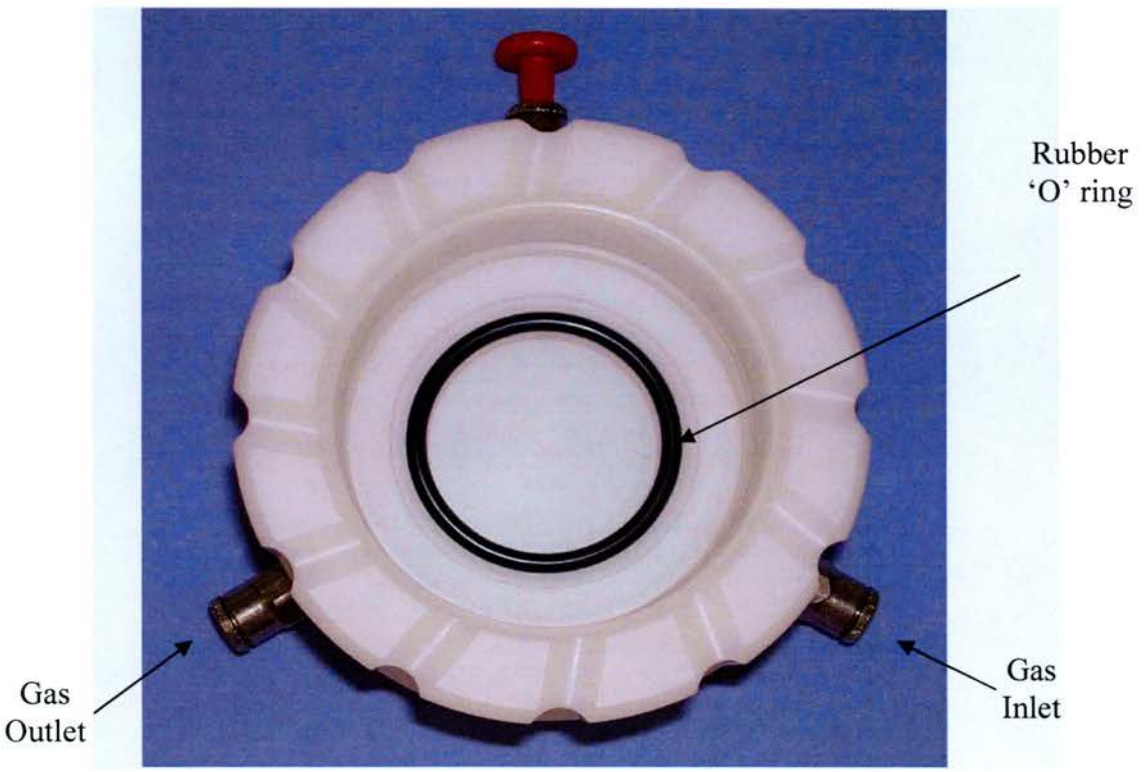


Figure 2.2 – Mechanical stimulation chambers

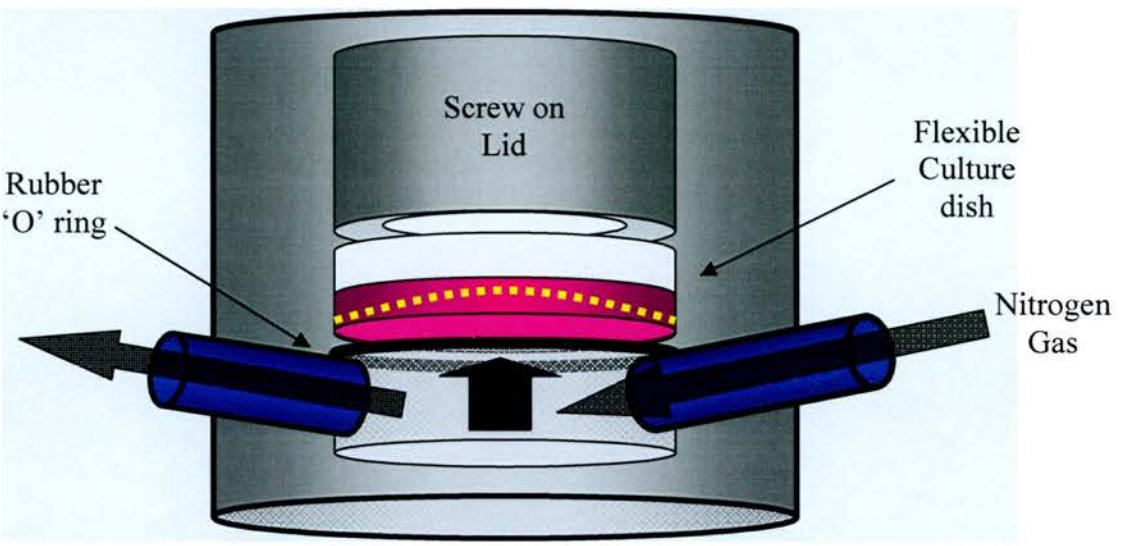


Figure 2.3 - Diagrammatic representation of mechanical stimulation chambers. Nitrogen gas enters the lower chamber causing increased pressure and resulting in the ballooning of the base of the dish upwards (represented by the dotted line).

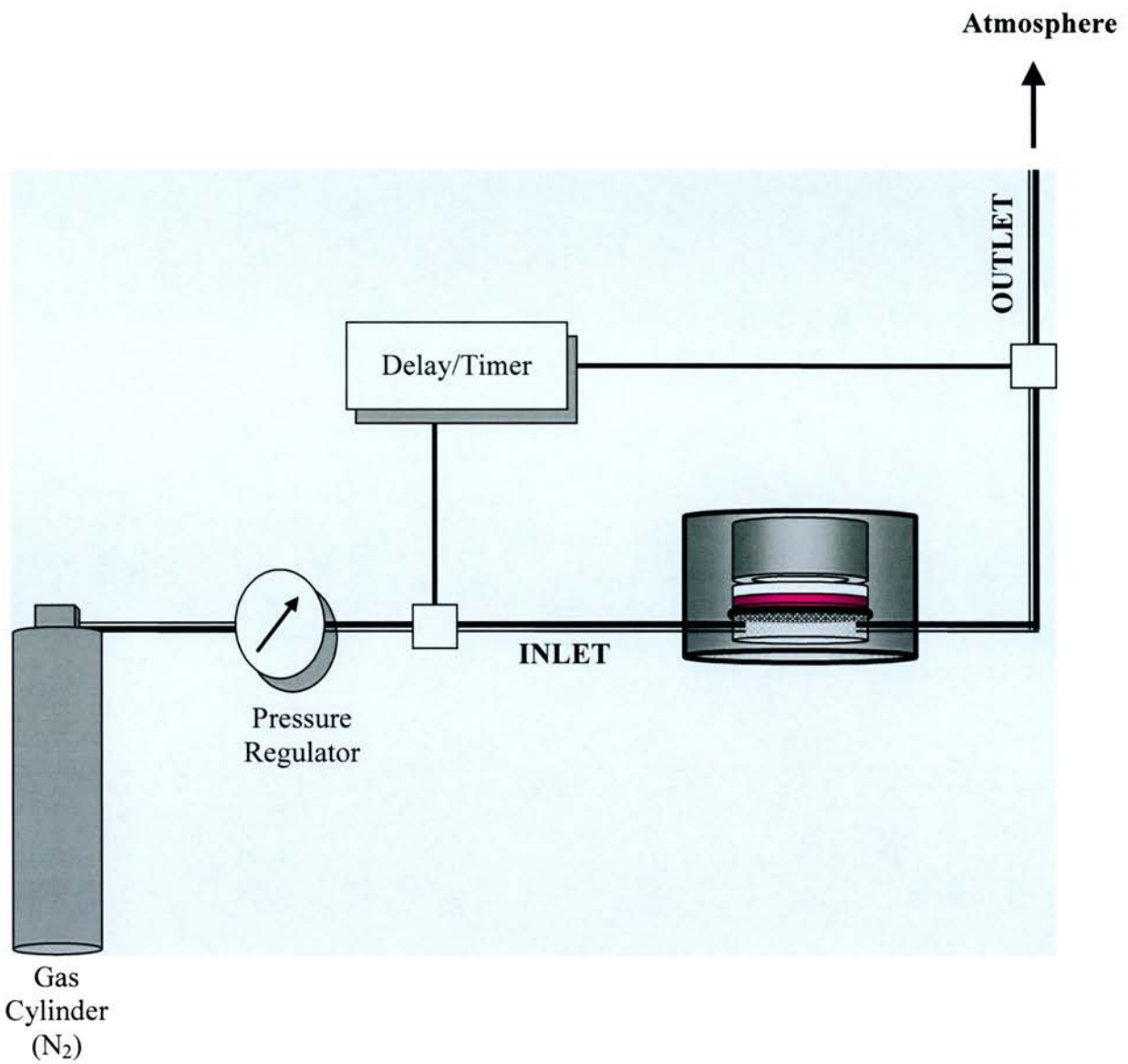


Figure 2.4 – Diagrammatic representation of mechanical stimulation apparatus

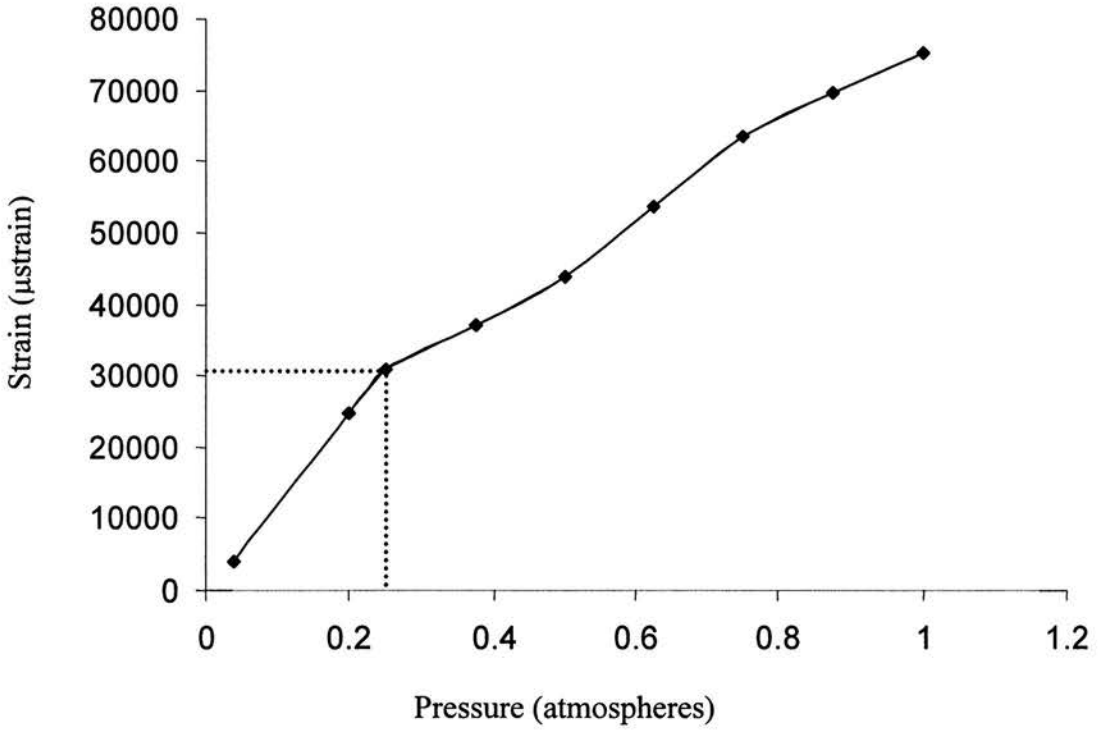


Figure 2.5 – Graph of strain calibration for mechanical stimulation system. A gas pressure of 0.25atmospheres (190mmHg) was used in these studies, resulting in ~31000μstrain on the base of the dish.

2.4 Chemical Reagents

To investigate the role of NOS in cartilage and the effects of mechanotransduction on NO production a variety of reagents were added to cell cultures. A cytokine cocktail was used to induce iNOS production with mechanical stimulation or iNOS inhibitor added simultaneously to determine their effects. The chemicals and concentrations used are detailed in **Table 2.3**.

	Reagent	Conc used	Other info	Supplier
Chemicals	iNOS inhibitor (AR-C102222)	1μM, 10μM, 100μM		AstraZeneca in house
	S-nitroso-N-acetyl-penicillamine (SNAP)	10μM, 100μM, 1mM	(NO donor)	Sigma
	IL-1β	10pg/ml, 100pg/ml, 500pg/ml, 2.5ng/ml, 10ng/ml		R&D systems (Abingdon, England)
Cytokine cocktail	IL-1β	0.5ng/ml		R&D systems
	TNFα	5ng/ml		R&D systems
	IL-6	1ng/ml		R&D systems
	IFNγ	10ng/ml		R&D systems
Antibodies	B1-integrin blocking antibody	1μg/ml	Clone JBIA	Chemicon
	Polyclonal IL-4 neutralising antibody	1μg/ml and 10μg/ml	P-615	Harlan sera-lab (Loughborough, England)
	Monoclonal IL-4 neutralising antibody	1μg/ml and 10μg/ml	Clone 34019.111	R&D systems

Table 2.3 – Chemical Reagents used in these studies

2.5 Cell Viability Assays

2.5.1 Acridine Orange staining

Acridine orange (Sigma) is a fluorescent nucleic acid stain (Carmichael et al 1980) that can be used in cells to detect apoptosis. HAC were stimulated with cyclical mechanical stimulation (MS) (in 58mm petri dishes) and the chemicals described in **Table 2.3** (in a 24 well plate) in serum free medium at 37°C, 95%O_2, 5%CO_2 for 4 and 12 hours. Following the incubation period the medium was removed and the wells washed gently using sterile PBS. 300µl acridine orange (100µg/ml in PBS) was added to each well and examined immediately under a fluorescence microscope for the presence of apoptotic nuclei.

2.5.2 MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazonium bromide] assay

The MTT assay is a colorimetric assay where mitochondrial succinate dehydrogenase catalyses conversion of MTT to a blue formazan product. The cells must be viable for this reaction to occur.

HAC in 96 well plates were incubated with the chemicals described in **Table 2.3** in triplicate using serum free medium at 37°C, 95%O_2, 5%CO_2 for 4 and 12 hours. Triton-X-100 (0.2%) was used as a positive control. The medium was removed after incubation and cells washed gently with PBS. MTT working solution was made up by adding 1.6ml of MTT stock (10mg/ml in PBS) (Sigma) to 5.4ml of serum free medium and 100µl added to each well and incubated for 3 hours in the dark at 37°C, 95%O_2, 5%CO_2. The MTT solution was then removed and cells washed in PBS before addition of 100µl dimethyl sulfoxide (DMSO) and gentle agitation for a few seconds to dissolve the formazan salt. The plate was then read at a wavelength of 630nm using a spectrophotometer within 30 minutes, with absorbance proportional to cell number.

HAC in 58mm petri dishes were incubated with the cytokine mix or IL-1β and subjected to MS for 4 and 12 hours before being treated in the same way as the 96 well plate above, except that volumes of MTT and DMSO added were 1ml.

2.6 RNA Purification

2.6.1 RNA extraction

HAC RNA extraction occurred immediately after the 4 and 12 hour experimental incubation times using a single culture dish for each condition tested. Dishes were placed on ice, the culture medium removed (1ml was kept to perform the Griess Assay) and the cells gently washed with sterile PBS. 350µl RNA extraction buffer RLT (Qiagen, Crawley, UK) plus β-mercaptoethanol (10µl/ml) (Sigma) was added directly to the cells. Plates were swirled gently for 5-10 minutes to ensure all cells were lysed, then scraped using a sterile cell scraper (Greiner, Shroudwater business park, Gloucestershire) and transferred to a DNase/RNase-free microfuge tube. The samples were mixed thoroughly using a pipette before being transferred to a QIAshredder spin column (Qiagen) and spun at 13000g for 2 minutes. 350µl 70% ethanol (Hayman, Witham, UK) was added to the homogenised lysate and mixed by pipetting. The sample was then transferred to an RNeasy mini column (Qiagen) and spun at 13000g for 15 seconds, discarding the flow-through. 350µl buffer RW1 (Qiagen) was added and spun for 15 seconds at 13000g with the flow-through being discarded. 80µl DNase I (Qiagen) was pipetted directly onto the silica-gel membrane and left at room temperature for 15 minutes before RW1 was again added and the sample spun at 13000g, with the flow-through being discarded. 500µl buffer RPE (Qiagen) was added to the spin column and spun for 15 seconds before adding further 500µl RPE and spinning for 2 minutes at 13000g. The flow through was discarded and the column placed in a new 1.5ml DNase/RNase-free microfuge tube. 30µl DEPC treated RNase-free water was added to the column before spinning for 1 minute at 13000g. The flow-through was pipetted back into the spin column and the spin repeated with the flow through stored at -70°C.

2.6.2 RNA Quantification

An Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech) was used to quantify the RNA. RNase-free water alone was used to zero the instrument and the absorbance of 1µl RNA in a total of 100µl was measured at both 260 and 280nm to obtain the DNA/RNA ratio.

2.7 Polymerase Chain Reaction (PCR) Methods

2.7.1 Reverse Transcription (RT) reactions

0.5ml Sterile RNAse/DNase-free tubes were used to perform RT reactions. Each RT reaction contained 1µg RNA, 200ng Random Hexamers (Promega, Southampton, UK) in a total volume of 11µl using RNase free water. The contents of the tube were mixed before being denatured by heating for 10 minutes at 70°C on an Omn-E™ thermal cycler (Hybaid, Teddington, UK). Following this 0.5mM 2'-Deoxynucleoside 5'-Triphosphates (dNTPs) (Promega), 10mM Dithiothreitol (DTT), 1xRT reaction buffer and 200U Superscript II RT (all Invitrogen) was added to each tube to give a final volume of 20µl.

The tubes were placed on the Omn-E™ thermal cycler for 50 minutes at 42°C and 70°C for 10 minutes. The synthesised cDNA was stored at -70°C until required.

2.7.2 Primer Design

The sequences used for PCR primers and probes (**Table 2.4**) were designed in-house (for this study or previously designed for other studies) using mRNA sequences from the Genbank database held at the National Centre for Biotechnology Information web site. Wherever possible primers were designed which were intron spanning to minimise the chances of genomic DNA contamination. All primers were synthesised by Applied Biosystems (ABI) (Warrington, UK) and designed using Primer Express™ v1.0 (Perkin Elmer) with a FAM label attached to the probes. 18s is the exception as it was purchased as a pre-made set (ABI), with the probe being VIC labelled.

Specificity	Description	Sequence (5'→3')
Aggrecan	Forward	GCC TGC GCT CCA ATG ACT
	Reverse	AAT GGA ACA CGA TGC CTT TCA
	Probe	GCC ATG CAT CAC CTC GCA GCG GTA T
iNOS	Forward	GCA GGT CGA GGA CTA TTT CTT TCA
	Reverse	CTT CGC CTC GTA AGG AAA TAC AG
	Probe	CTC AAG AGC CAG AAG CGC TAT CAC GAA G
eNOS	Forward	CCC GCT CGA GGC ACA
	Reverse	TGT TTG GCC GAG TCC TCA
	Probe	CCC CAA GAC CTA CGT GCA GGA CAT C
nNOS	Forward	GGC CGA AGC TCC AGA ACT C
	Reverse	GTG TGG AGA CGC ACG AAG ATA G
	Probe	TCC AGA GCC CTA AAT CCA GTC GGT CA
Mouse Aggrecan	Forward	ACT CCC ACA GTT GGC AGG TT
	Reverse	CAG CCC ACT AAG GTC TCC TGT T
	Probe	CCT TCG GGG CTC TCA CCT CCA GAG

Table 2.4 – Primer and Probe sequences used in these studies

Primer/Probe set	Probe added (5μM stock)	Primer added (10μM stock)	RNase-free water (μl)
18s	0.25μl (10μM stock)	0.625μl (20μM stock)	7.125
Aggrecan	0.5μl	0.125μl	6.75μl
iNOS	0.5μl	0.125μl	6.75μl
eNOS	0.5μl	0.75μl	5.5μl
nNOS	0.5μl	2.25μl	2.5μl
Mouse Aggrecan	0.5μl	0.125μl	6.75μl

Table 2.5 – Primer and Probe concentrations used in these studies

2.7.3 PCR Protocol

PCR reactions were performed using 96 well plates made up of 0.2ml RNase/DNase free tubes with a final reaction volume of 25µl. A typical reaction contained 12.5µl 2x Mastermix (containing dNTPs, Hot Goldstar DNA polymerase, $MgCl_2$, Uracil-N-glycosylase) (Eurogentec, Romsey, UK), 0.125µl forward primer (10µM), 0.125µl reverse primer (10µM), 0.5µl probe (5µM), 5µl cDNA and 6.75µl RNase-free water. Details of the primer/probe concentrations used in each reaction are in **Table 2.5**.

2.7.4 PCR Programme

The samples were run on an ABI PRISM^{TM} 7700 Sequence Detector (Perkin Elmer). Thermal cycler conditions were in three stages: Stage 1 was 50°C for 2 minutes, Stage 2 was 95°C for 10 minutes and Stage 3 included 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fluorescent release from the probe was recorded in real time by an attached computer using the Sequence Detection Systems v1.6.3 alias software (Perkin Elmer). Threshold cycle (C_T) values from experimental samples were converted to relative mRNA levels using a standard curve of dilutions of plasmid cDNA coding for the NOS isoforms (**Figure 2.6**)

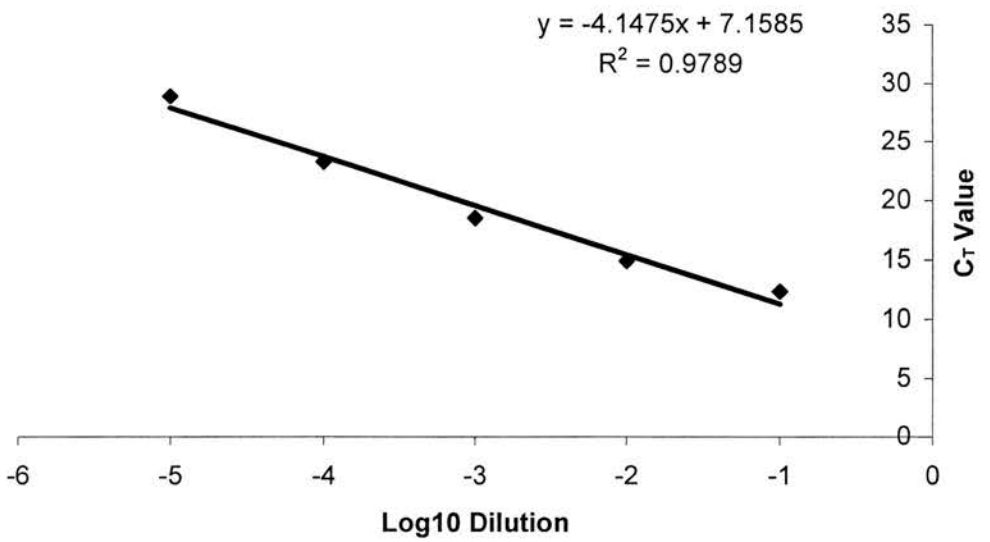


Figure 2.6 – Taqman RT-PCR standard curve of iNOS plasmid cDNA dilutions against Threshold cycle (C_T) value to allow calculation of relative mRNA levels in experimental samples.

2.8 Protein Purification – Western Blotting

2.8.1 Protein Extraction

HAC protein extraction occurred immediately after the 4 or 12hour experimental incubation times with a single culture dish used for each condition tested. Dishes were placed on ice, the culture medium removed (1ml was kept to perform the Griess assay) and the cells were gently washed with sterile PBS. 300µl protein extraction buffer containing 50mM HEPES (pH 7.4) (Sigma), 1mM EDTA (Sigma), 0.1mM DTT (Sigma), 20mM CHAPS (Calbiochem, Nottingham, UK) and complete mini protease inhibitor cocktail (Roche, Lewes, UK) was added directly to the cells. Plates were kept on ice and swirled occasionally for 15-20 minutes to ensure all cells were lysed, then scraped using a sterile cell scraper (Greiner, Gloucestershire, UK) and transferred to a microfuge tube. The samples were mixed thoroughly using a pipette, subjected to centrifugation (13000g for 15 minutes) and the supernatant was transferred to a fresh microfuge tube and the protein concentration was quantified.

2.8.2 Protein Assay

The protein concentration was quantified using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Perbio Science, Cramlington, UK). The assay is based on the biuret reaction, where protein reduces Cu^{2+} to Cu^{1+} in an alkaline medium. The Cu^{1+} binds BCA producing a purple colour that can be read at 540nm (Smith 150, 76 (1985)). Dilutions of a 2mg/ml bovine serum albumin (Sigma) solution were used to construct a standard curve and 20µl of this and each of the samples was added to a 96 well plate in duplicate. Reagents A and B from the BCA kit were mixed (50:1) and 200µl added to each well. The plate was covered and incubated at 37°C for 30 minutes before reading at 540nm, with absorbance being relative to protein concentration.

2.8.3 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The Bio-Rad (Hemel Hempstead, UK) Protein II xi cell system was used to carry out SDS-PAGE with resolving and stacking gels made up as shown in **Table 2.6** and **Table 2.7** respectively.

20μg total protein was mixed with a half volume of 2xloading buffer made up of 5ml 0.375M Tris (pH6.8), 4.5ml dH_2O, 8ml 10%SDS, 2ml glycerol, 0.4ml β-mercaptoethanol and 150μl of a saturated bromophenol blue solution and put in a boiling water bath for 5 minutes. Samples were then cooled on ice and loaded into the gel in a gel tank filled with 4 litres of electrode buffer containing 25mM Tris, 190mM glycine and 0.1%SDS (all Sigma) in dH_2O. Gels were run at 100volts for 35 minutes and then 200volts for 3 hours using a Bio-Rad Powerpac 3000. *E. coli* derived NOS isoforms, DLD-1 (iNOS) and EAhy926 (eNOS) cell lysates were used as positive controls (**Table 2.8**) and Full Range Rainbow™ molecular weight marker (Amersham, Chalfont St. Giles, UK).

Reagent	Volume
Acrylamide (30%) (Amresco)	13.5ml
1M Tris (pH8.85) (Sigma)	15ml
10% SDS (Sigma)	0.4ml
dH_2O	11.1ml
TEMED (Sigma)	0.1ml
10%Ammonium Persulphate (AMPS) (Sigma)	0.1ml

Table 2.6 – Resolving gel (10%)

Reagent	Volume
Acrylamide (30%) (Amresco)	3.6ml
0.375M Tris (pH6.8) (Sigma)	10ml
10% SDS (Sigma)	0.3ml
dH_2O	16ml
TEMED (Sigma)	0.1ml
10%AMPS (Sigma)	0.1ml

Table 2.7 – Stacking gel (3.6%)

Source	Control	Conc used
E.coli lysate	iNOS	10µl 1:100 stock dilution
E.coli lysate	eNOS	10µl 1:50 stock dilution
E.coli lysate	nNOS	20µl 1:20 stock dilution
DLD-1 (cytokine stimulated)	iNOS	50µg total protein
EAhy926	eNOS	50µg total protein

Table 2.8 – Western blot positive controls

2.8.4 Transfer

Hybond ECL Nitrocellulose membrane (Amersham) was cut to size and soaked for 15minutes in transfer buffer containing 25mM Tris, 190mM glycine and 0.005%SDS (all Sigma) in dH_2O. Blotting paper and sponges were also soaked in transfer buffer and each cassette set up as shown in **Figure 2.7**.

Transfer was carried out overnight at 15V and 4°C and then at 25V, 4°C for 1 hour the following morning. A Bio-Rad Transblot cell with plate electrodes and a Bio-Rad model 200/2.0 power supply were used.

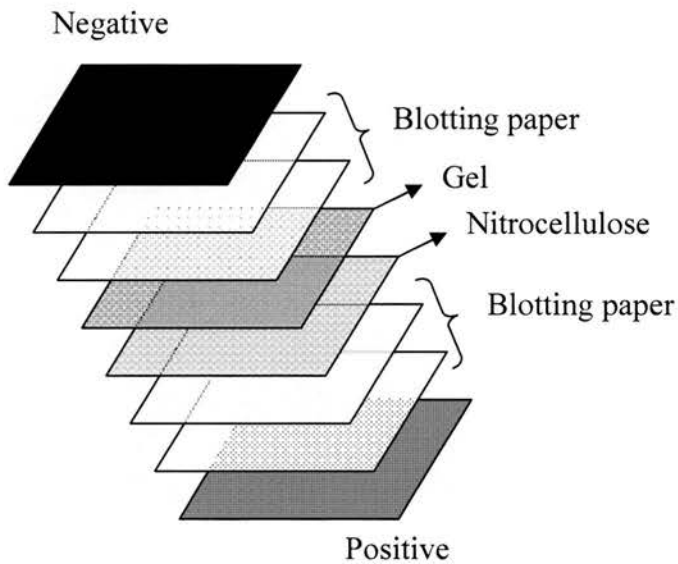


Figure 2.7 – Diagrammatic representation of component orientation in Transfer cassette

2.8.5 Probing Membrane

The nitrocellulose membrane was blocked for 1 hour at room temperature using a antibody dilution buffer containing 200ml TBS, 10g Dried milk and 0.2ml Tween-20 with gentle agitation applied using a bench top roller. Primary antibody diluted in antibody dilution buffer was added to the membrane and incubated at room temperature for 1 hour using the dilutions shown in **Table 2.9**. This was then washed in Tris buffered Saline containing 20mM Tris (sigma) and 137mM NaCl (sigma) at pH7.6 plus 0.1%Tween-20 (TBS-T) for 10 seconds, 30 seconds, 5 minutes and 10 minutes before the addition of HRP-linked secondary antibody at the dilutions shown in **Table 2.9** for 1 hour at room temperature. The 4 washes in TBS-T were repeated before ECL+ (Amersham) was added for 3 minutes and then the membrane dripped dry for 10 seconds. The membrane was loaded into a film cassette and taken up to the dark room where x-ray film (Kodak) was set on top of the membrane, exposing it to the presence of any fluorescing bands. The film was then developed using a Hyperprocessor (Amersham).

2.8.6 Stripping membrane

In order to re-probe the same membrane for different proteins of interest the membranes were stripped of the attached antibodies. This was carried out using a stripping buffer containing 100mM β-mercaptoethanol, 2%SDS, 62.5mM Tris-HCl (pH6.7) and incubated for 30 minutes at 50°C with occasional agitation. The membrane was then washed using large volumes of TBS-T at room temperature before proceeding with blocking and probing the membrane as described above.

Primary Antibody Specificity	Primary Antibody info	Dilution used	Secondary Antibody used	Secondary Antibody Info	Secondary Antibody dilution
iNOS (Santa Cruz)	H-174 (sc-8310)	1:1000	HRP-linked donkey anti rabbit IgG	NA 934 (Amersham)	1:2000
eNOS (Sigma)	aa596-609 (N 2643)	1:10,000	HRP-linked donkey anti rabbit IgG	NA 934 (Amersham)	1:2000
nNOS (Sigma)	NOS-B1 (N 2280)	1:3000	HRP-linked sheep anti mouse IgG	NA 931 (Amersham)	1:2000
GAPDH (Abcam, Cambridge, UK)	6C5 (ab8245)	1:12,500	HRP-linked sheep anti mouse IgG	NA 931 (Amersham)	1:2000

Table 2.9 – Antibody details and dilutions used in these studies

2.9 Nitrite Determination

In order to measure the level of NO produced during the experiment the level of its stable degradation product nitrite (NO_2^-) is measured using the Griess Reagent (Sigma). This is a modification of a diazotization reaction originally described by Griess in 1879. This is a 2 step reaction where nitrite reacts with sulphanilamide initially and the product of this reacts with N-1-napthylethylenediamine dihydrochloride under acidic (phosphoric acid) conditions to produce an Azo compound (**Figure 2.8**). 100µl of post-experimental conditioned medium is added to 100µl of Griess reagent and the generation of azo-product can be measured at a wavelength of 550nm, correlating to nitrite levels. Dilutions of a Sodium nitrite 100µM solution were used to create a standard curve.

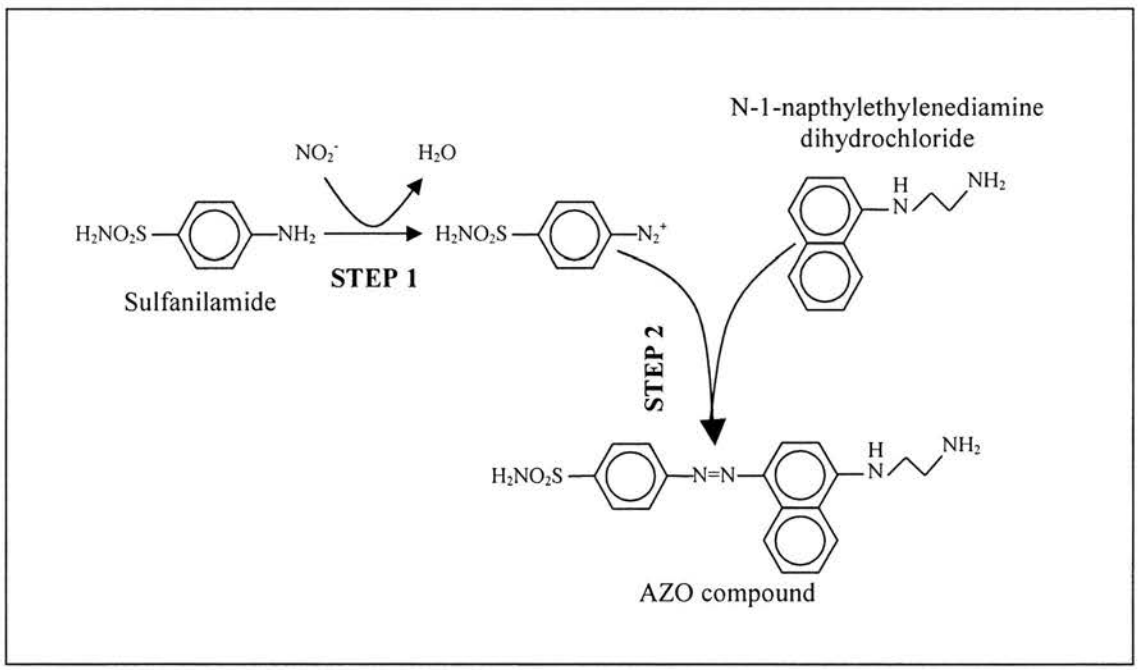


Figure 2.8 – Griess assay 2 step diazotization reaction to measure nitrite levels

2.10 – Statistics

The mean, standard deviation and standard error of the mean (SEM) were determined in each experiment. Variances were assessed by comparing the F-ratio of the standard deviations for each pair of values. Where the variance was not significantly different results were assessed using an unpaired t test. When the F-ratio of the two variances reached significance the Mann-Whitney U test was used to assess the data which was not parametrically distributed. In a number of instances where responses in individual samples followed a closely similar pattern results were compared using a paired t test. Results were considered statistically significant when the p value was less than 0.05.

Performing multiple T-tests can cause problems as the more tests that are performed the higher the chance of there being an inaccurate test. It is for this reason that an adjustment or correction factor may be beneficial where a number of T-tests have been performed. In order to avoid this overuse of the T-test it may be more appropriate to use a different statistical method where differences between two or more groups can be assessed using a single test, such as ANOVA.

Chapter 3 – Transformed chondrocyte Nitric Oxide Synthase (NOS) isoform expression

Aims

- Investigate NOS isoform expression in chondrocyte cell lines
- Investigate the effects of cytokine stimulation and mechanical stimulation on NOS isoform expression in chondrocyte cell lines.

3.1 - Introduction

The production of nitric oxide (NO) is known to have many effects on the regulation of cartilage matrix integrity, as discussed in Section 1.3.6. The cells responsible for cartilage regulation are the chondrocytes, and the presence and activity of enzymes that produce NO within these cells are therefore of interest. In this study two transformed chondrocyte cell lines, C20A4 and C28I2, have been analysed for the presence of all three enzymes known to produce NO, endothelial (e), neuronal (n) and inducible (i) nitric oxide synthase (NOS). The effect of cyclic mechanical stimulation at 0.33Hz and 30,000μstrain (MS) and proinflammatory cytokine cocktail stimulation (CYT) on NOS expression and activity has also been studied. NOS mRNA levels were detected using Taqman real time PCR and protein levels investigated by Western blotting. The activity of the NOS enzymes was determined using a Griess assay where the stable end product of NO, nitrite, is measured.

3.2 – Results of Transformed Chondrocyte Cell line NOS isoform production

3.2.1 – Cell Line NOS Isoform mRNA Detection

Taqman real-time PCR was used to detect the relative levels of the three NOS isoforms in both C20A4 and C28I2 cells. This technique involves the synthesis of cDNA from mRNA in the experimental samples, followed by the amplification of cDNA for forty identical cycles with the release of fluorescence recorded in real time as the cycles proceed (**Section 2.7**). The level of mRNA in the original sample is calculated using the number of cycles required for cDNA amplification to reach the log phase, known as the Threshold cycle (C_T) value. A dilution series of plasmid cDNA coding for the NOS isoforms is used to generate a standard curve by plotting their C_T values against the dilution factor. Relative mRNA levels from the experimental samples are then calculated from the standard curve.

eNOS mRNA was detectable following 36 cycles in unstimulated samples from both cell lines. eNOS mRNA was detected at cycle 35 and 36 in C20A4 and cycle 36 and 38 in C28I2 following the application of 4 and 12 hour MS respectively. Relative eNOS levels calculated from these C_T values and adjusted for the 18s content (**Figure 3.1**) show slightly elevated levels after 4 hours MS in C20A4 and decreased levels after 12 hours MS in C28I2 when compared to unstimulated controls. Relative eNOS mRNA levels following CYT (**Figure 3.1**) show decreased levels at 4 hours and increased levels at 12 hours in C20A4, while increased levels were seen at both 4 and 12 hours in C28I2. eNOS mRNA was detected at cycle 36 and 35 in C20A4 and cycle 35 and 37 in C28I2 following the simultaneous application of MS and CYT at 4 and 12 hours respectively. Relative eNOS mRNA levels following CYT plus MS (**Figure 3.1**) showed a slight increase at 4 hours and a decrease at 12 hours compared to CYT alone in both cell lines.

nNOS mRNA was detectable at cycle 37 in C20A4, and cycle 33 in C28I2 unstimulated cells. nNOS mRNA levels are detected from cycle 38 to 40 following the application of MS, CYT and CYT plus MS at both 4 and 12 hours in C20A4. nNOS mRNA levels are detected at cycle 34 following MS, CYT and CYT plus MS at 4 hours in C28I2. At 12 hours nNOS mRNA levels are detected at cycle 34

following MS and at cycle 37 following both CYT and CYT plus MS in C28I2. Relative nNOS levels calculated from these C_T values (**Figure 3.2**) are higher in the C28I2 than C20A4 cells. CYT and MS appear to reduce relative nNOS levels from unstimulated controls at 4 and 12 hours in both cell lines, while MS has little effect on the nNOS mRNA levels seen following CYT.

iNOS mRNA was detected in unstimulated cells from cycle 39 in C20A4 and cycle 33 in C28I2. C20A4 iNOS mRNA was detected at cycles 36 and 32 following 4 and 12 hours CYT respectively. C28I2 iNOS mRNA was detected at cycles 30 and 29 following 4 and 12 hours CYT respectively. Relative iNOS mRNA levels calculated from these C_T values (**Figure 3.3**) following MS are slightly increased from unstimulated controls after 4 hours, and decreased after 12 hours in both cell lines. CYT elevated relative iNOS mRNA levels at both 4 and 12 hours in both cell lines, with MS increasing this effect after 4 hours, while decreasing it after 12 hours.

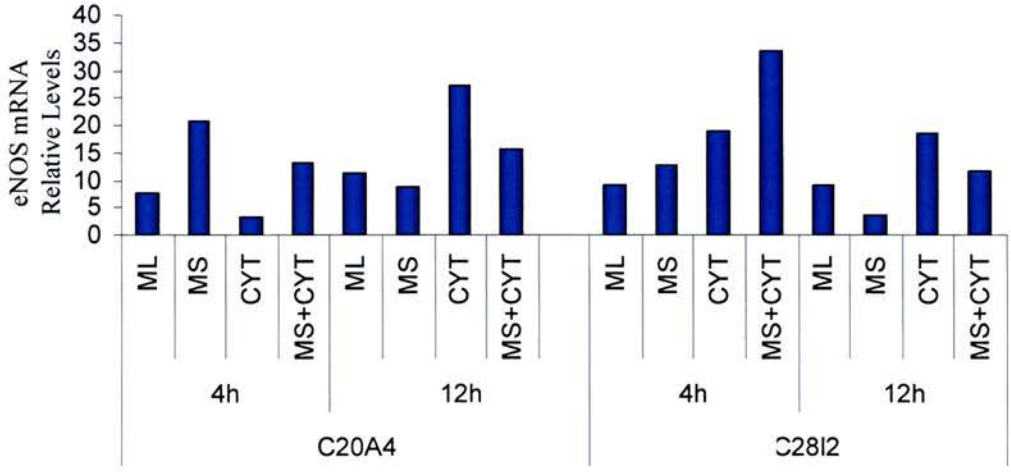


Figure 3.1 – Taqman PCR analysis of eNOS mRNA levels in transformed chondrocytes C20A4 and C28I2. Relative levels were evaluated at 4 and 12 hours of culture with no stimulation (ML), Mechanical Stimulation (MS), Cytokine cocktail stimulation (CYT) and Mechanical plus Cytokine stimulation (CYT+MS). (n=1)

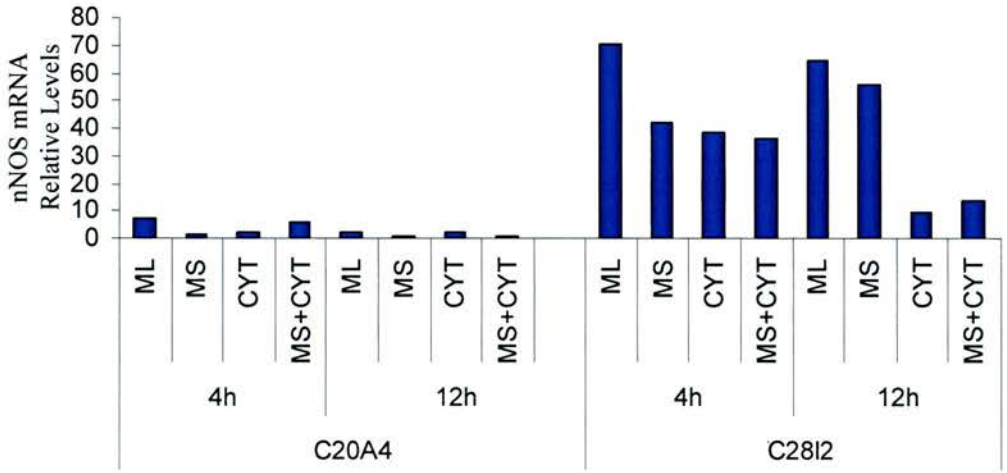


Figure 3.2 – Taqman PCR analysis of nNOS mRNA levels in transformed chondrocytes C20A4 and C28I2. Relative levels were evaluated at 4 and 12 hours of culture with no stimulation (ML), Mechanical Stimulation (MS), Cytokine cocktail stimulation (CYT) and Mechanical plus Cytokine stimulation (CYT+MS). (n=1)

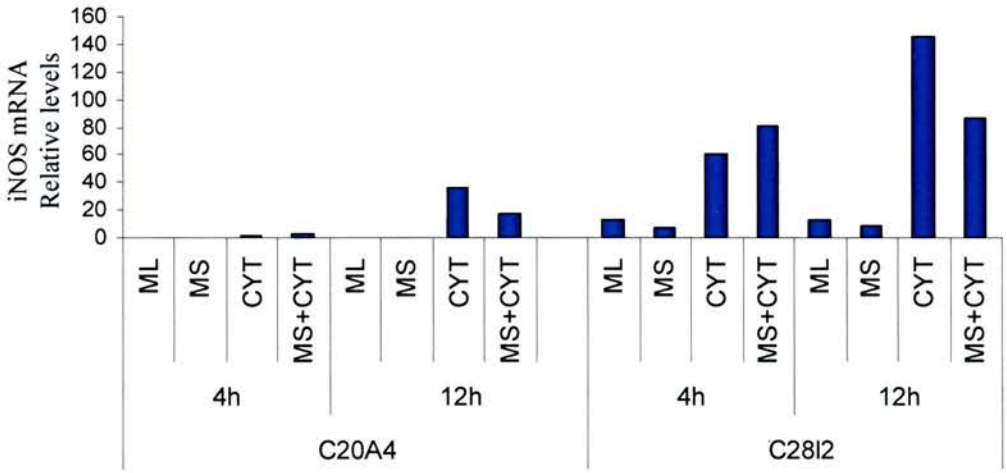


Figure 3.3 – Taqman PCR analysis of iNOS mRNA levels in transformed chondrocytes C20A4 and C28I2. Relative levels were evaluated at 4 and 12 hours of culture with no stimulation (ML), Mechanical Stimulation (MS), Cytokine cocktail stimulation (CYT) and Mechanical plus Cytokine stimulation (CYT+MS). (n=1)

	C20A4				C28I2			
	ML	MS	CYT	MS+CYT	ML	MS	CYT	MS+CYT
eNOS	C_T=37	↑4hr ↓12hr	↑12hr	↑4hr ↓12hr	C_T=37	↑ 4hr ↓12hr	↑4hr ↑12hr	↑4hr ↓12hr
nNOS	C_T=37	↓ 4hr ↓ 12hr	↓4hr ↓12r	↓4hr ↓12hr	C_T=32	↓ 4hr ↓ 12hr	↓4hr ↓12hr	↓4hr ↓12hr
iNOS	C_T=37	↑4hr ↓12hr	↑ 4hr ↑ 12hr	↑4hr ↓12hr	C_T=33	↑4hr ↓12hr	↑ 4hr ↑ 12hr	↑4hr ↓12hr

Table 3.1 – Summary of NOS mRNA levels in transformed chondrocytes C20A4 and C28I2. Levels were evaluated at 4 and 12 hours of culture with no stimulation (ML), Mechanical Stimulation (MS), Cytokine cocktail stimulation (CYT) and Mechanical plus Cytokine stimulation (CYT+MS). (n=1)

3.2.2 – Cell Line NOS Isoform Protein Detection

Having detected small amounts of mRNA for the three NOS isoforms in the two chondrocyte cell lines, Western blotting was used to determine whether any protein was detectable. Western blotting was performed using specific antibodies for all 3 NOS isoforms, with GAPDH detected as a measure of protein loading. Appropriate controls for each of the NOS isoforms, and the use of cytokine stimulated primary chondrocyte cell lysate show that the blot is working well and if these proteins were present they would have been detected. However, no protein was detected in either cell line for any of the three NOS isoforms (**Figures 3.4 and 3.5**).

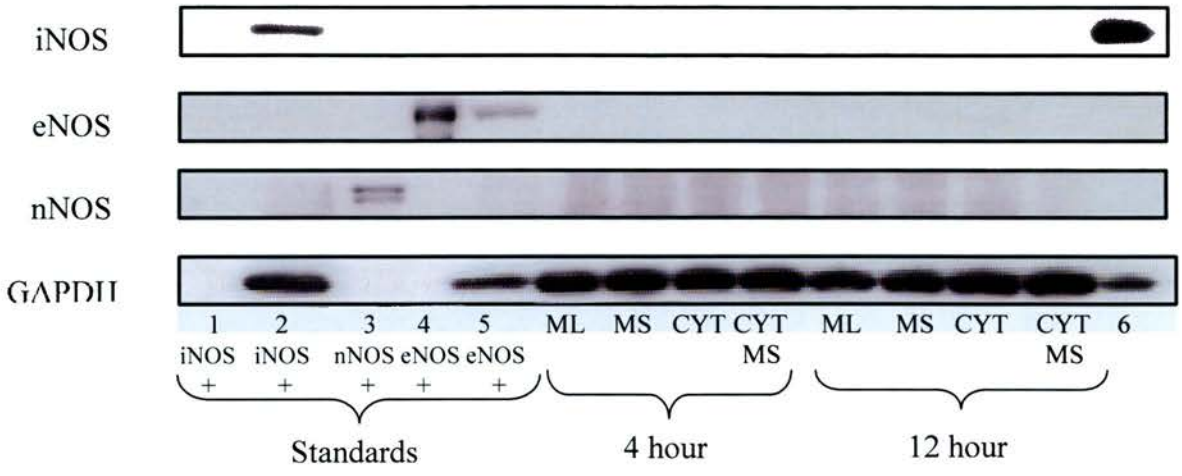


Figure 3.4 – Western Blot for NOS isoforms in C20A4 transformed chondrocytes. Protein levels were evaluated at 4 and 12 hours with no stimulation (ML), Mechanical Stimulation (MS), Cytokine cocktail stimulation (CYT) and Mechanical plus Cytokine stimulation (CYT+MS). Lane 1 = iNOS *E. coli* lysate 2 = CYT stimulated DLD-1 3 = nNOS *E. coli* lysate 4 = eNOS *E. coli* lysate 5 = Eahy926 eNOS 6 = CYT stimulated primary HAC. (n=3)

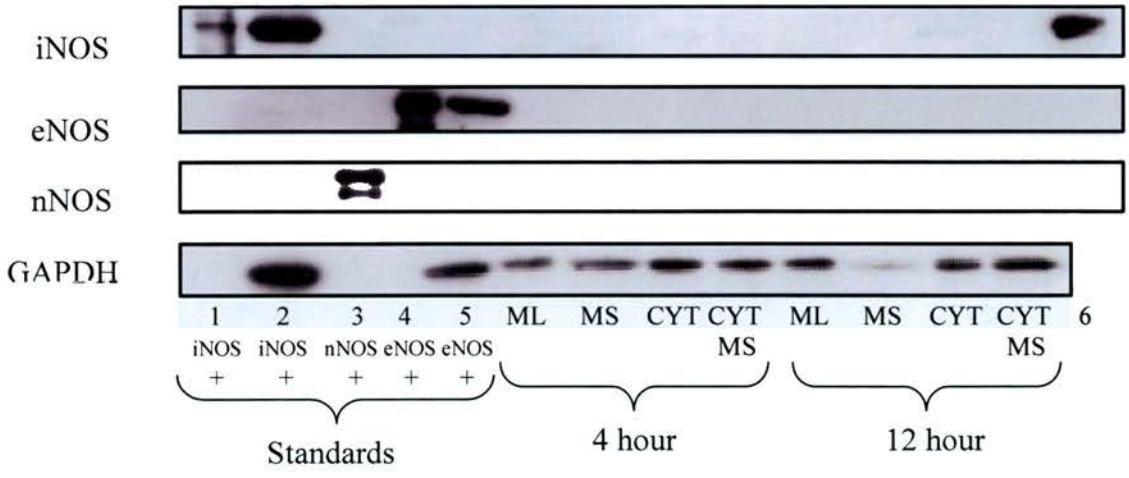


Figure 3.5 – Western Blot for NOS isoforms in C28I2 transformed chondrocytes. Protein levels were evaluated at 4 and 12 hours with no stimulation (ML), Mechanical Stimulation (MS), Cytokine cocktail stimulation (CYT) and Mechanical plus Cytokine stimulation (CYT+MS). Lane 1 = iNOS *E. coli* lysate 2 = CYT stimulated DLD-1 3 = nNOS *E. coli* lysate 4 = eNOS *E. coli* lysate 5 = Eahy926 eNOS 6 = CYT stimulated primary HAC. (n=3)

3.2.3 – Cell Line Nitrite Production

The Griess assay was performed to identify whether there was any NOS activity detectable in the chondrocyte cell lines. Medium was collected at 4 and 12 hours from resting, CYT stimulated, MS, and CYT plus MS, and analysed for nitrite production. A standard curve constructed from dilutions of sodium nitrite in medium allowed calculation of the concentration of nitrite in each sample, and acted as a control to show the assay was working. Very small alterations in the absorbance at 540nm were detected, translating into low levels of nitrite at the extremes of the detection limits of the assay (**Figure 3.6**).

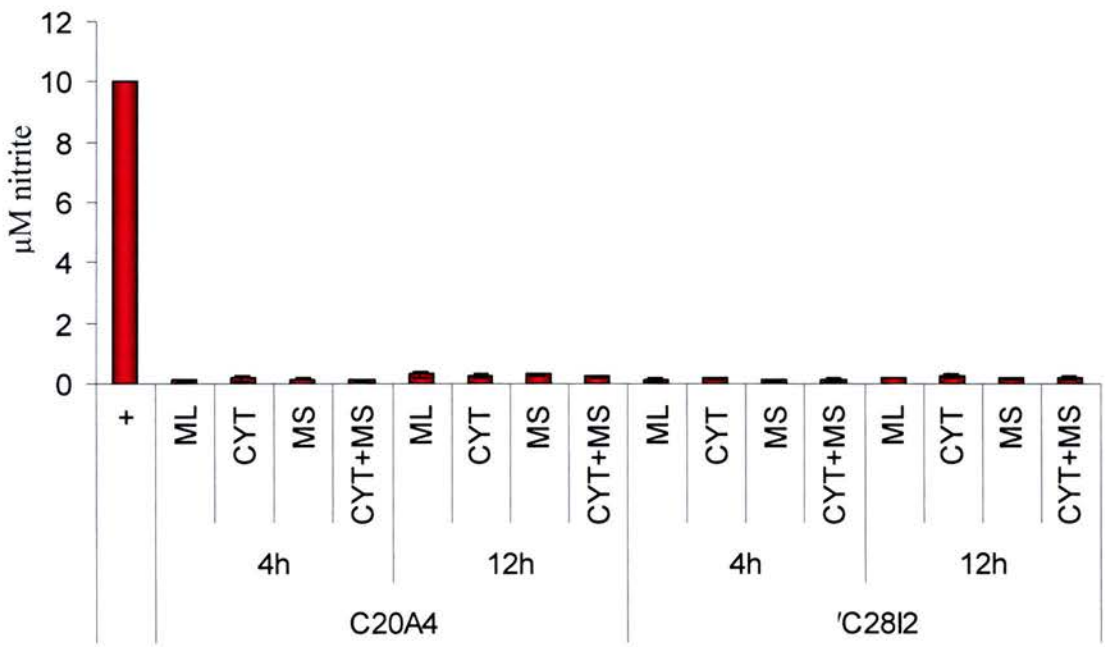


Figure 3.6 – Griess assay to determine levels of nitrite produced by both transformed cell lines at 4 and 12 hours with no stimulation (ML), Mechanical Stimulation (MS), Cytokine cocktail stimulation (CYT), Mechanical plus Cytokine stimulation (CYT+MS) and Sodium nitrite standard curve value (+). (n=3)

3.3 – Discussion

3.3.1 – Findings

The results obtained show that mRNA is detected for all three NOS isoforms in the transformed chondrocyte cell lines. Alterations in mRNA expression were seen following mechanical and cytokine stimulation as well as differences in the levels detected between the two cell lines (summarised in Table 3.1). It must be stated, however, that these data come from a single experiment so it is not possible to draw any conclusions about the variation in these levels without increasing the number of replicates.

It must also be noted that in many cases these values are near the end of the amplification procedure and indicate that very little NOS isoform mRNA was present within the original sample. This means that the calculation of relative mRNA levels may show several fold increases in values, while the actual changes in mRNA levels are unlikely be significant.

The western blot and Griess assay experiments were repeated on a number of occasions, confirming that NOS protein and enzyme activity is not seen in the transformed chondrocyte cell lines under any of the conditions tested.

3.3.2 - Interpretation of results

The detection of iNOS mRNA but the lack of iNOS protein and enzyme activity implicates factors involved in mRNA transcription, translation or protein degradation of iNOS in the lack of cytokine induced NO production.

Transcriptional regulation of the iNOS gene can occur at the level of synthesis, stability and degradation (summarised in **Figure 1.6**). However, the fact that iNOS mRNA is detected in these cell lines following cytokine stimulation suggests transcription takes place and it may be translational events that are responsible for the lack of iNOS protein and enzyme activity.

The presence of four AUUUA repeats present in the 3'-UTR of the iNOS mRNA (Geller et al 1993) which have been shown to confer destabilization to cytokine and oncogene mRNAs may be responsible for a lack of iNOS translation (Caput et al 1986). Several proteins can bind this region and alter its stability, these may be important in controlling the level of iNOS transcription in our studies.

There also remains the possibility that the mRNA transcripts that are detected by the Taqman RT-PCR technique are not full length and could be alternatively spliced but contain the region amplified by this technique (Eissa et al 1998). This could account for the elevated mRNA levels seen and for the lack of protein transcribed.

3.3.3 – iNOS expression in primary chondrocytes

Primary chondrocytes are one of the few cell types that produce iNOS in response to single cytokine stimulation, including IL-1β or TNFα (Grabowski et al 1996; Palmer et al 1993). IL-6 and IFNγ have not been shown to independently induce iNOS, but act in synergy with IL-1β and TNFα to increase iNOS production in chondrocytes (Henrotin et al 2000; Huang et al 2004; Kinugawa et al 1997; Tuna et al 2001). The proinflammatory cytokine cocktail used in these studies is therefore a potent stimulator of iNOS production in primary human chondrocytes, and thus may be expected to induce similar levels in the transformed chondrocytes.

3.3.4 – Factors that may have influenced NOS expression

The experiments in this chapter were carried out using the immortalized chondrocyte cell lines C20A4 and C28I2 (Goldring et al 1994a). These are derived from costal chondrocytes from a five year old male and a fifteen year old female respectively.

SV40 large T antigen transfection was used to immortalize the chondrocytes, stabilising their proliferative capacity (Wolff et al 1992). This transfection may influence iNOS production. Indeed, there are several reports of other cell lines that are either unaffected (Horton et al 1998: Liu et al 1993: Murakami et al 2002) or show decreased iNOS production (Aldieri et al 2004) following SV40 transformation.

Immortalised chondrocytes have previously been shown to be highly proliferative but express little collagen type II, a marker for cell differentiation (Block et al 1991). Growth in three dimensional agarose constructs was used to induce differentiation during development of these cell lines. However, when expanded in monolayer the maintenance of phenotype decreased with time (Goldring et al 1994b). The importance of maintenance of the differentiated phenotype is highlighted by the fact that C20A4 cells only produce COXII after IL-1 stimulation when they have been in 3-d alginate culture for six weeks (Goldring and Berenbaum 1999; Robbins et al 2000).

3.4 – Conclusion

These transformed chondrocytes show several of the responses seen in primary cells and can display a differentiated phenotype under the correct conditions. In a single experiment NOS isoform mRNA was detected, but no conclusions can be drawn from this data without repeat studies. These cells do not produce iNOS protein and enzyme activity following cytokine stimulation, however, suggesting that they have altered transcription or translation patterns. The response of these cells may be influenced by many factors including the transformation, differentiation, age and gender of the donor cartilage. In addition to altered response to cytokine signalling, alterations in the actin cytoskeleton (Brown and Benya 1988) and integrin expression pattern (Loeser et al 2000) seen in transformed chondrocytes will influence the way these cells sense and respond to mechanical stimuli.

Low amounts of available primary cells and difficulties with *in vivo* models suggest that, provided the limitations are understood and results obtained are validated with primary cells or cartilage explants, the system remains a useful research tool. The results in this chapter, however, indicate the cell lines are of limited use for the study of the effects of NO on chondrocytes.

Summary of findings

- The transformed chondrocyte cell lines C20A4 and C28I2 show detectable levels of e, n and iNOS mRNA, however these data come from a single experiment

- C20A4 and C28I2 do not produce NOS isoform protein or nitrite following 4 or 12 hour incubation with proinflammatory cytokines or mechanical strain.

Chapter 4 – The effects of cyclical mechanical stimulation on NOS levels in primary human articular chondrocytes

Aims

- Investigate NOS isoform expression in primary osteoarthritic human articular chondrocytes

- Investigate the effects of proinflammatory cytokine stimulation, mechanical stimulation and a novel iNOS inhibitor on NOS isoform expression in primary osteoarthritic human articular chondrocytes

4.1 - Introduction

In order to investigate the level of nitric oxide synthase (NOS) enzymes in primary human articular chondrocytes (HAC) Taqman real-time PCR was used to measure mRNA levels, and western blotting using NOS specific antibodies was used to detect protein levels. NOS enzyme activity was determined using the Griess assay, measuring the levels of the stable nitric oxide product, nitrite, in the medium.

Primary HAC were initially stimulated by either the proinflammatory cytokine cocktail containing IL-1β, TNFα, IFNγ and IL-6 (CYT) or IL-1β alone and the effect of concentration on NOS expression was evaluated. The effect of cyclical mechanical stimulation at 0.33Hz and 30,000μstrain (MS) on both CYT and IL-1β stimulated iNOS levels and activity was studied. The method by which MS may be interacting with CYT and IL-1β induced iNOS was investigated using IL-4 neutralising antibodies and a β1 integrin function blocking antibody. The effect of a novel iNOS inhibitor, AR-C102222, on iNOS levels and activity was also evaluated.

4.2 – Results

4.2.1 – NOS mRNA, protein and nitrite production in primary OA HAC

eNOS, nNOS and iNOS mRNA were detected in unstimulated HAC after 32, 37 and 28 cycles of Taqman PCR respectively. iNOS mRNA levels were measured in primary HAC after 12 hours incubation with CYT. iNOS mRNA levels were detected from cycle 23 following incubation with both half and full concentration of CYT, calculated as a 50.7 fold increase in relative levels at half and a 139.4 fold increase at full CYT ($p=0.1$) (**Figure 4.1**). CYT stimulation reduced the basal levels of eNOS mRNA by 64% (**Figure 4.2**) and nNOS mRNA by 93% (**Figure 4.3**) in the primary HAC, although these values were not statistically significant.

iNOS protein levels were not detectable at 12 hours in unstimulated primary HAC or after half CYT stimulation, but were seen following full CYT stimulation as determined by western blot (**Figure 4.4**). Neither e nor nNOS proteins were detected in primary HAC prior to, or following CYT incubation (**Figure 4.6**).

Nitrite levels were detected at 0.2μM in unstimulated cells, which is at the limits of detection for the assay. Nitrite levels were measured at 4.7μM following half CYT ($p=0.1$) and 5.6μM following full CYT ($p=0.1$) stimulation in primary HAC (**Figure 4.4**).

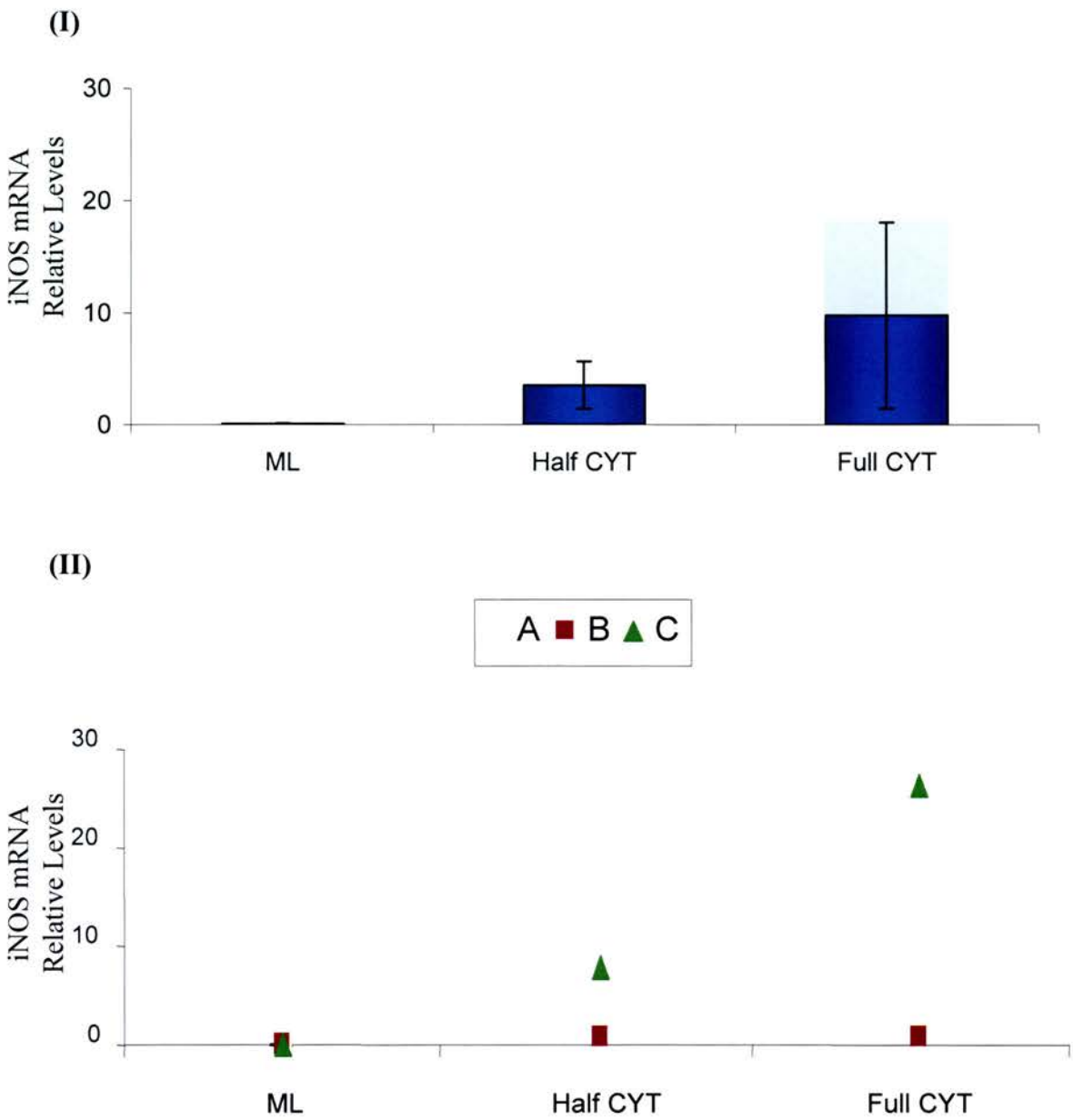


Figure 4.1 – Basal (unstimulated) and cytokine cocktail stimulated iNOS mRNA levels (12hr). (I) = Pooled values±SEM. (II) = Individual experimental values. ML = Unstimulated control. Half CYT = Half concentration of cytokine cocktail. Full CYT = Cytokine cocktail stimulation. A=7321 B=7329 C=7488. (n=3)

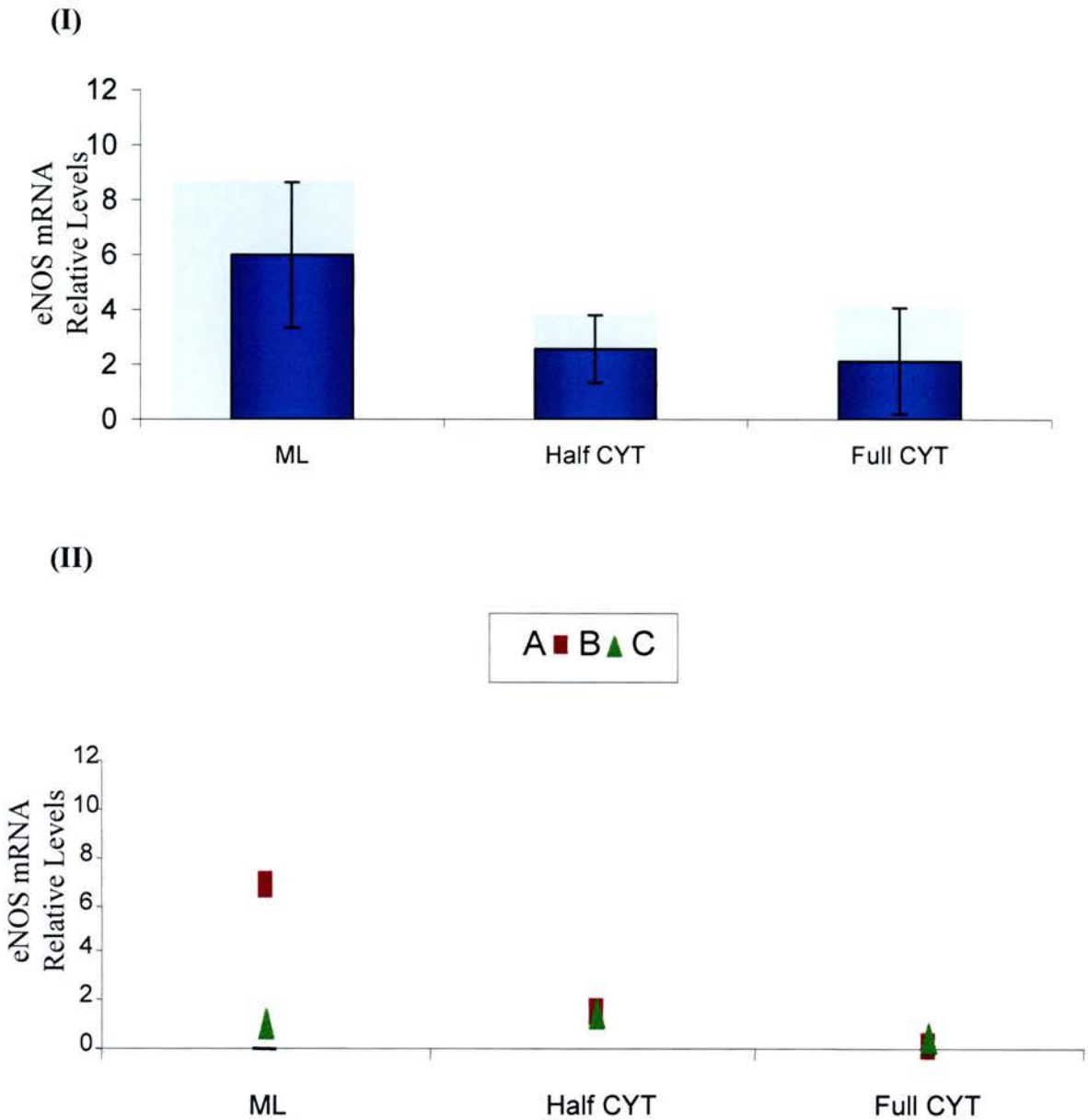


Figure 4.2 – Basal (unstimulated) and cytokine cocktail stimulated eNOS mRNA levels (12hr). (I) = Pooled values±SEM. (II) = Individual experimental values. ML = Unstimulated control. Half CYT = Half concentration of cytokine cocktail. Full CYT = Cytokine cocktail stimulation. A=7321 B=7329 C=7488. (n=3)

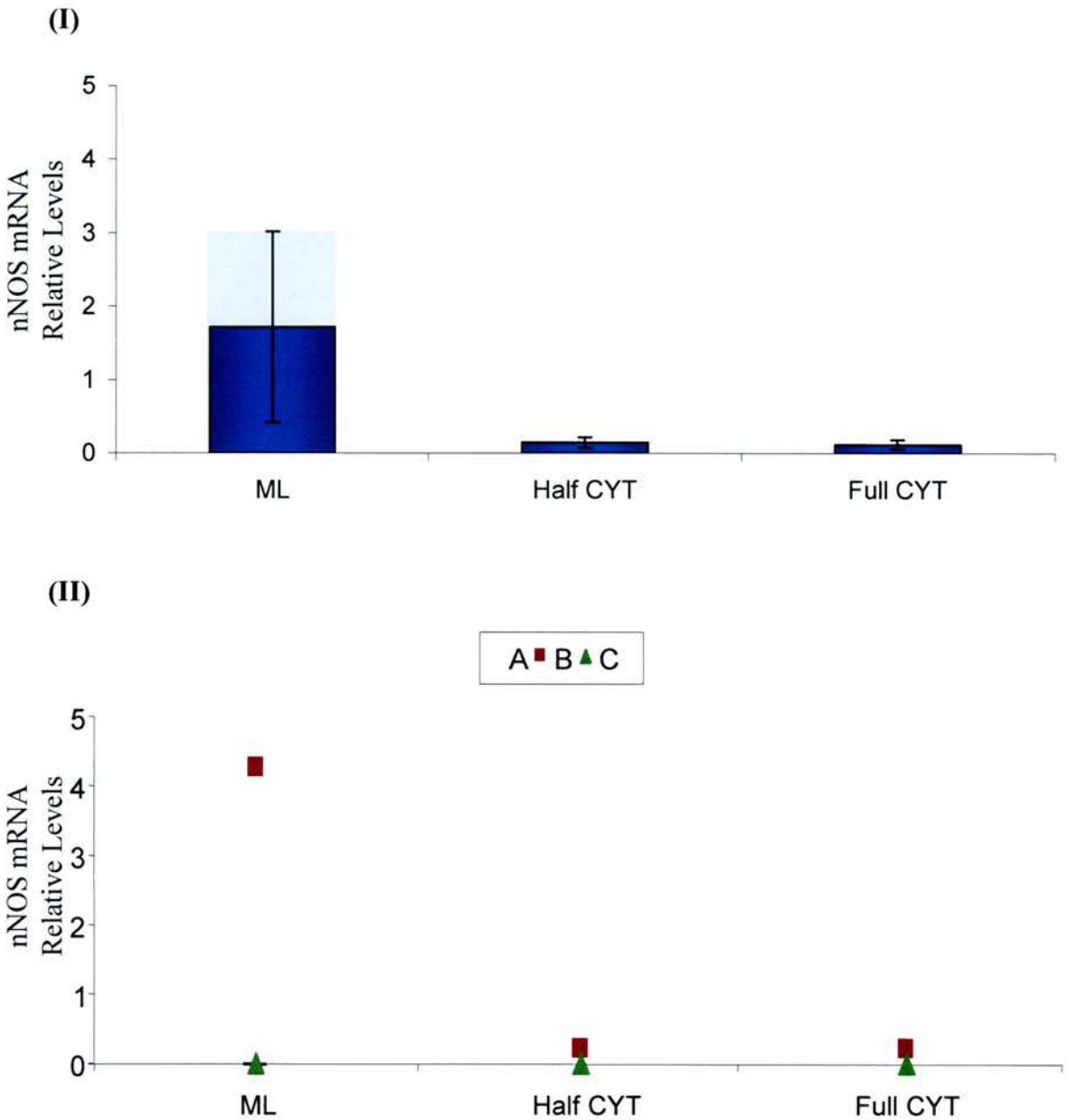
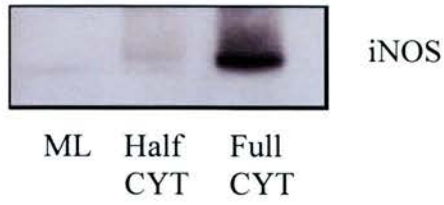
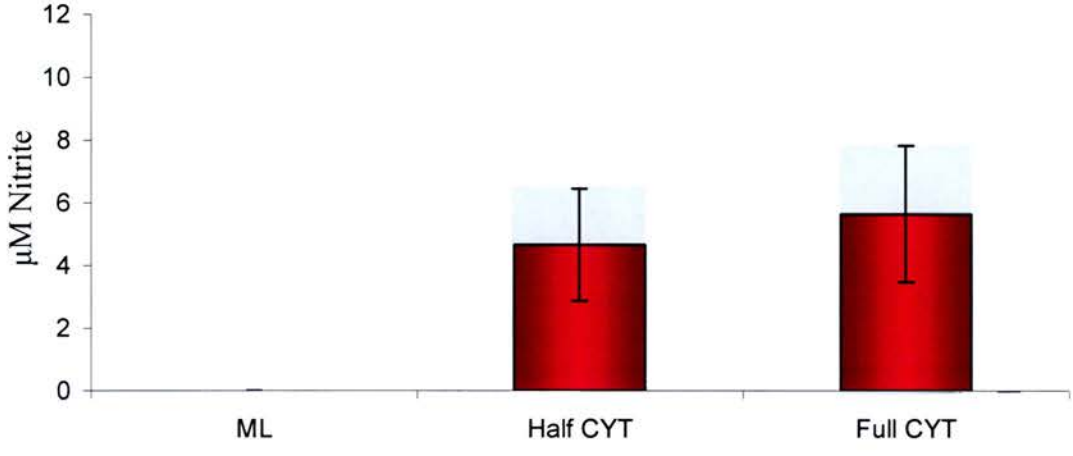


Figure 4.3 - Basal (unstimulated) and cytokine cocktail stimulated nNOS mRNA levels (12hr). (I) = Pooled values±SEM. (II) = Individual experimental values. ML = Unstimulated control. Half CYT = Half concentration of cytokine cocktail. Full CYT = Cytokine cocktail stimulation. A=7321 B=7329 C=7488. (n=3)

(I)



(II)



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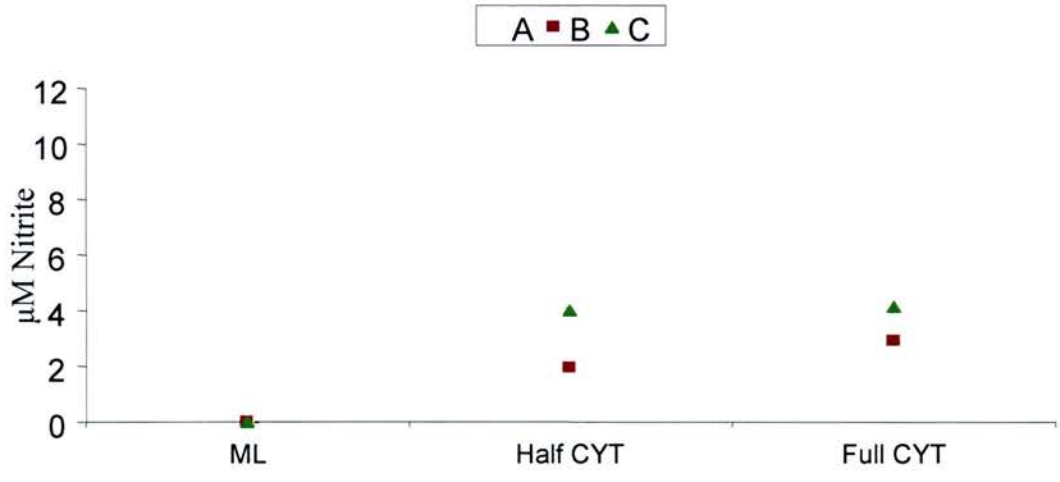


Figure 4.4 - iNOS protein and nitrite levels prior to and following cytokine cocktail stimulation (12hr). (I) = Western blot probing for iNOS. (II) = Griess assay pooled nitrite levels±SEM. (III) = Griess assay individual experimental values. ML = Unstimulated control. Half CYT = Half concentration of cytokine cocktail. Full CYT = Cytokine cocktail stimulation. A=7321 B=7329 C=7488. (n=3)

4.2.2 – Effects of cyclic mechanical stimulation on CYT induced iNOS

The levels of iNOS mRNA and protein, as well as nitrite accumulation in the medium, were measured following 4 and 12 hours of CYT and MS.

MS reduced unstimulated iNOS mRNA levels by 96% and 79% after 4 and 12 hours respectively, but this decrease was not significant. CYT stimulation significantly increased iNOS mRNA levels above unstimulated controls by 16 fold and 432 fold after both 4 and 12 hours respectively ($p<0.0001$) (**Figure 4.5**). The simultaneous application of CYT and MS also showed significantly elevated levels of iNOS mRNA above the level detected in the unstimulated control, 7.2 fold at 4 and 60.1 fold at 12 hours ($p=0.0002$ and $p<0.0001$ respectively). The iNOS mRNA level following the simultaneous application of CYT and MS was 55% lower after 4 hours and 86% lower after 12 hours than following CYT alone ($p=0.0014$ and $p<0.0001$ respectively) (**Figure 4.5**).

iNOS protein was not detected in unstimulated controls or after MS. CYT stimulation significantly increased the level of iNOS protein above unstimulated levels at both 4 and 12 hours ($p<0.0001$). Simultaneous stimulation with CYT and MS resulted in significantly higher iNOS mRNA levels than in unstimulated controls at both 4 and 12 hours ($p<0.0001$). iNOS protein levels following the simultaneous application of both CYT and MS were 70% lower after 4 hours and 76% lower after 12 hours, than following CYT alone ($p=0.04$ and $p=0.0003$ respectively) (**Figure 4.6**).

There was little nitrite production after 4 hours under any of the conditions tested (**Figure 4.7**). Nitrite was detected at 0.6 and 0.9μM at 12 hours in unstimulated controls and following MS alone respectively, which is at the limits of detection for the assay. Following 12 hour CYT there was a significant increase in nitrite levels, with concentrations of 6 and 7.5μM nitrite detected for mRNA and protein adjusted experiments respectively ($p<0.0001$). Nitrite concentrations following the simultaneous application of both CYT and MS were significantly higher than in unstimulated controls, but protein- and mRNA-adjusted levels were 58% and 63% lower respectively than following CYT alone ($p=0.0014$ and $p=0.0006$ respectively).

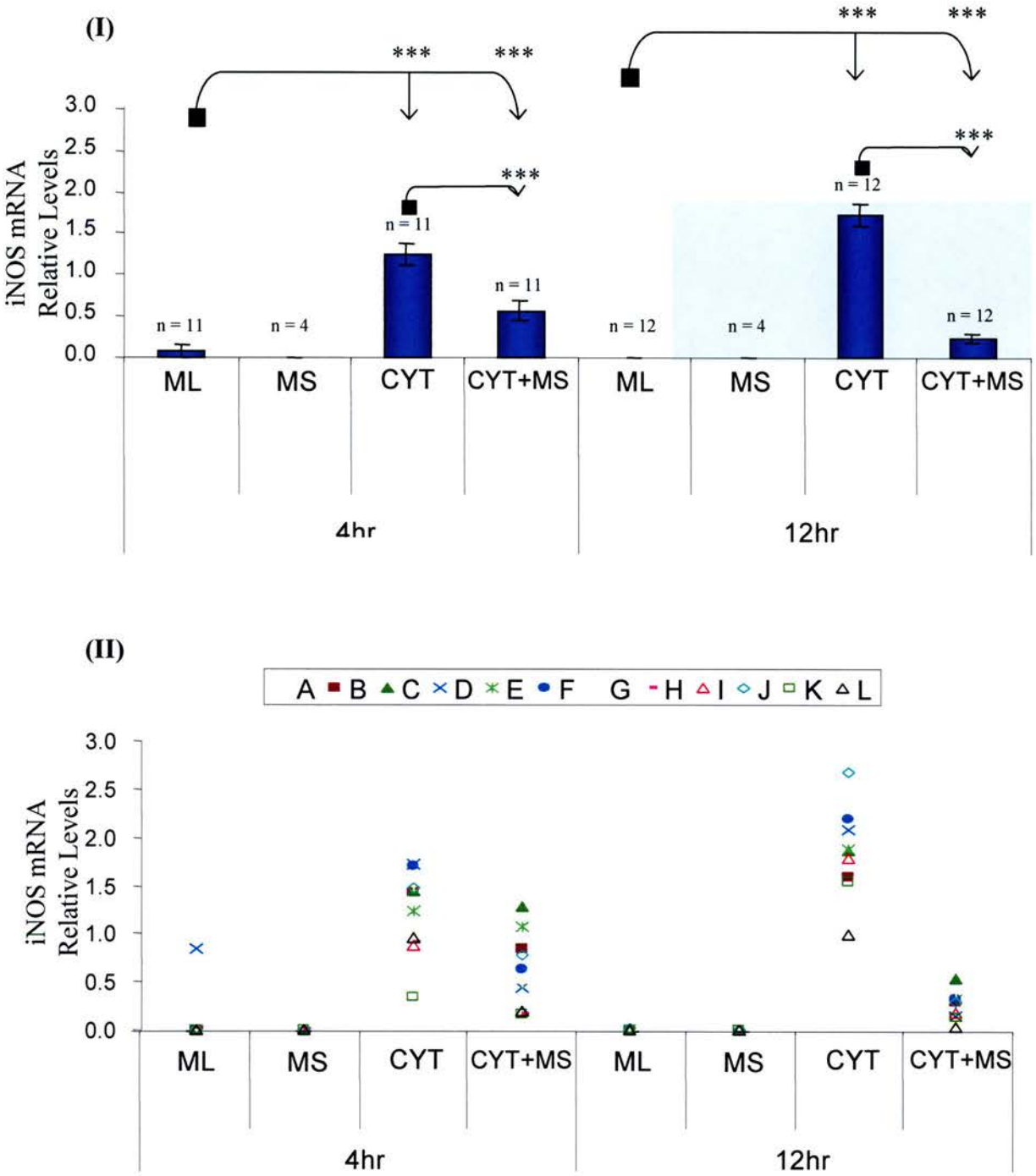


Figure 4.5 – Effect of mechanical stimulation (MS) on cytokine cocktail induced iNOS mRNA levels. (I) = Pooled iNOS mRNA levels±SEM. (II) = Individual experimental values. ML = Unstimulated control. MS = Mechanical stimulation. CYT = Cytokine cocktail stimulation. CYT+MS = Both cytokine cocktail and mechanical stimulation. A=3/10/03 B=18/4/04 C=29/3/04 D=22/3/04 E=8/3/04 F=10/2/04 G=18/4/04 H=6/12/02 I=17/6/03 J=22/6/03 K=27/6/03 L=28/6/03. *** $p<0.001$.

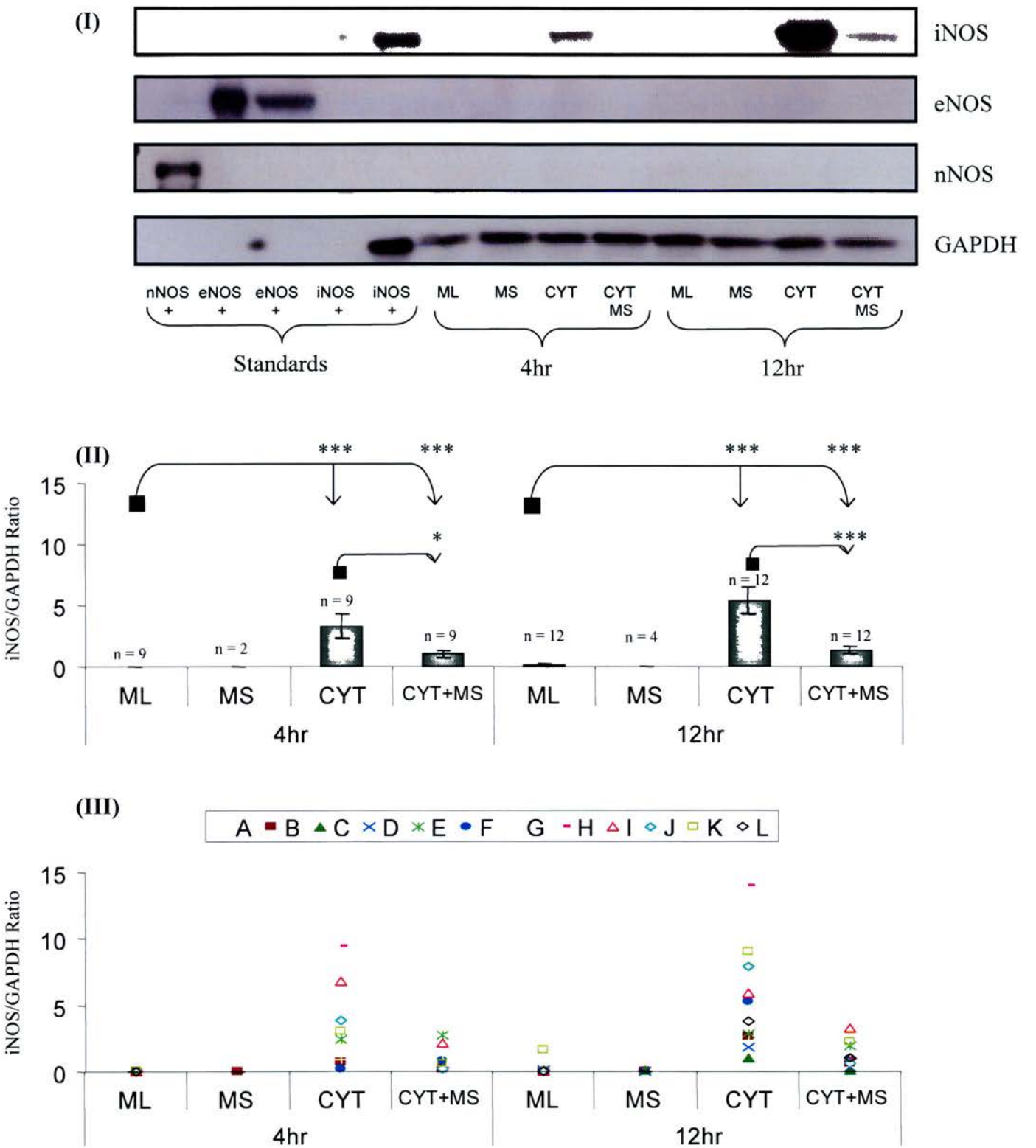


Figure 4.6 – Effect of MS on CYT induced iNOS protein levels. (I) = Western blot for NOS isoforms (28/6/03). (II) = Densitometry of Western blot bands pooled data±SEM. (III) = Densitometry individual experimental values. ML = Unstimulated control. MS = Mechanical stimulation. CYT = Cytokine cocktail stimulation. CYT+MS = Both cytokine cocktail and mechanical stimulation. A=17/6/03 B=28/6/03 C=6/12/02 D=DS008/9c E=10/2/04 F=14/2/04 G=22/3/04 H=3/10/03 I=11/11/03 J=23/9/03 K=5/9/03 L=3/9/03. * $p<0.05$ ***$p<0.001$.

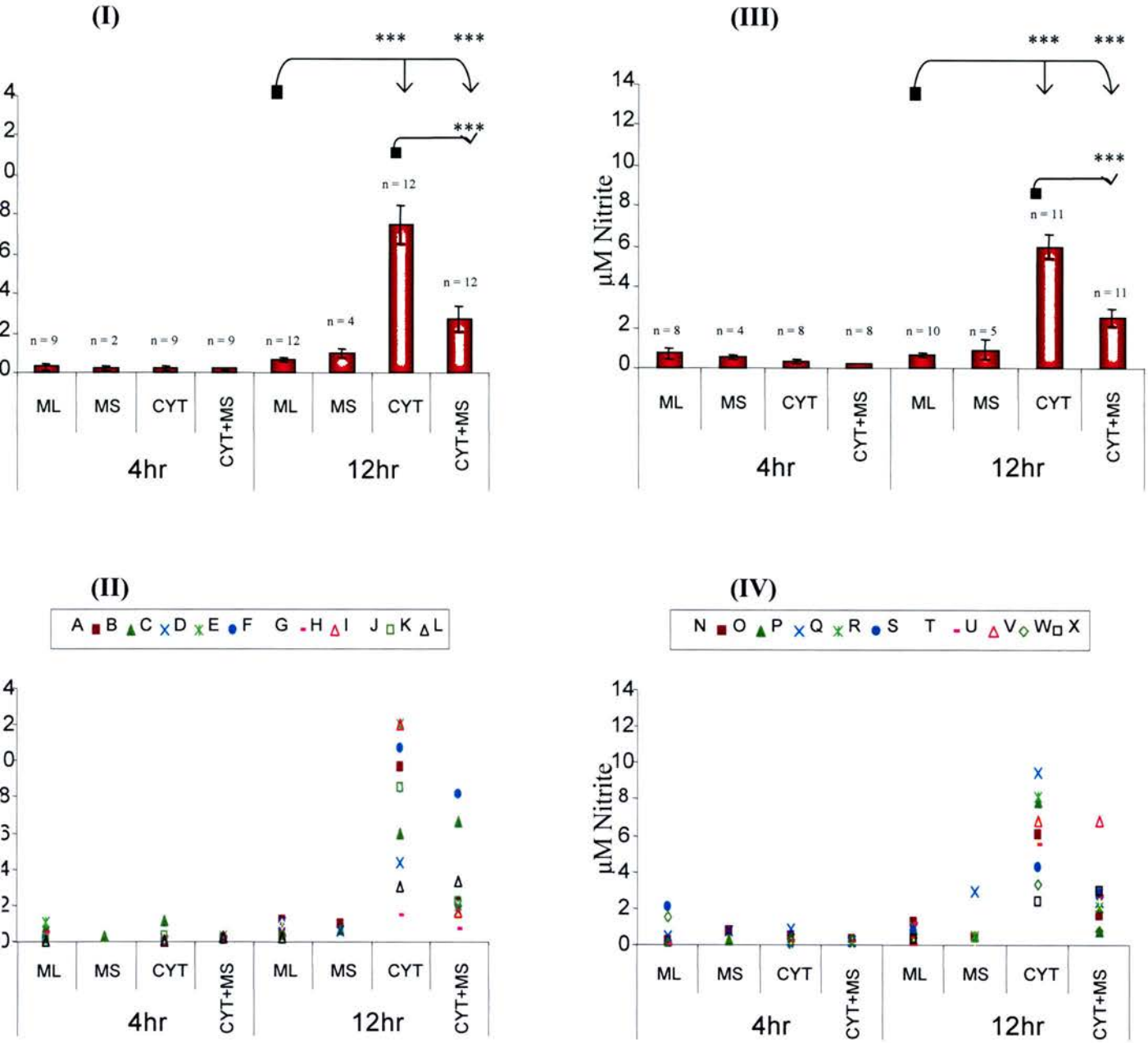


Figure 4.7 – Effect of MS on CYT induced nitrite levels. (I) = Griess assay adjusted according to the quantity of protein in the original sample. Pooled data±SEM. (II) = Protein adjusted Griess assay individual experimental values. (III) = Griess assay adjusted according to the quantity of mRNA in the original sample. Pooled data±SEM. (IV) = mRNA adjusted Griess assay individual experimental values. ML = Unstimulated control. MS = Mechanical stimulation. CYT = Cytokine cocktail stimulation. CYT+MS = Both cytokine cocktail and mechanical stimulation. A=28/6/03 B=DS008/9c C=17/6/03 D=6/12/02 E=22/3/04 F=10/2/04 G=14/2/04 H=3/10/03 I=23/9/03 J=11/11/03 K=3/9/03 L=5/9/03 N=22/6/03 O=27/6/03 P=28/6/03 Q=17/6/03 R=6/12/02 S=22/3/04 T=8/3/04 U=10/2/04 V=18/4/04 W=3/10/03 X=29/3/04. *** $p<0.001$.

4.2.3 – Effects of MS and an IL-4 neutralising antibody on CYT induced iNOS,

4.2.3a – Polyclonal IL-4 Neutralising Antibody

iNOS protein and nitrite levels were measured following CYT stimulation, and the effects of MS in the presence of a polyclonal IL-4 neutralising antibody at low (1μg/ml) and high (10μg/ml) concentrations were evaluated.

iNOS protein levels were increased following CYT stimulation at both 4 and 12 hours (**Figure 4.8**). The simultaneous application of CYT and MS also resulted in increased iNOS protein levels compared to unstimulated controls, but 79% lower at 4 hours and 74% lower at 12 hours than following CYT alone. The presence of 10μg/ml of the polyclonal IL-4 neutralising antibody in combination with CYT stimulation was associated with 66% lower iNOS protein levels after 12 hours, than following CYT stimulation alone. There was a 76% lower iNOS protein level in cells stimulated with CYT plus MS in the presence of 10μg/ml polyclonal IL-4 neutralising antibody than following CYT plus MS. The presence of the antibody at a concentration of 1μg/ml does not influence the level of iNOS protein detected following CYT or CYT plus MS.

After 4 hours CYT stimulation nitrite levels are at 0.1μM in the culture medium, which is at the limits of detection for the assay. Nitrite levels were increased to 5.7μM following 12 hours CYT stimulation (**Figure 4.9**). The simultaneous application of CYT and MS increased nitrite levels to 2.7μM at 12 hours, 52% lower than following CYT alone. The presence of the polyclonal IL-4 neutralising antibody at 10μg/ml resulted in reduced CYT-induced nitrite levels, from 5.7μM to 1.4μM, and decreased concentrations following the simultaneous application of CYT and MS (from 2.7μM to 0.3μM). The lower concentration of the polyclonal IL-4 neutralising antibody did not influence nitrite levels.

These experiments were carried out on only two occasions so statistical analysis can not be performed.

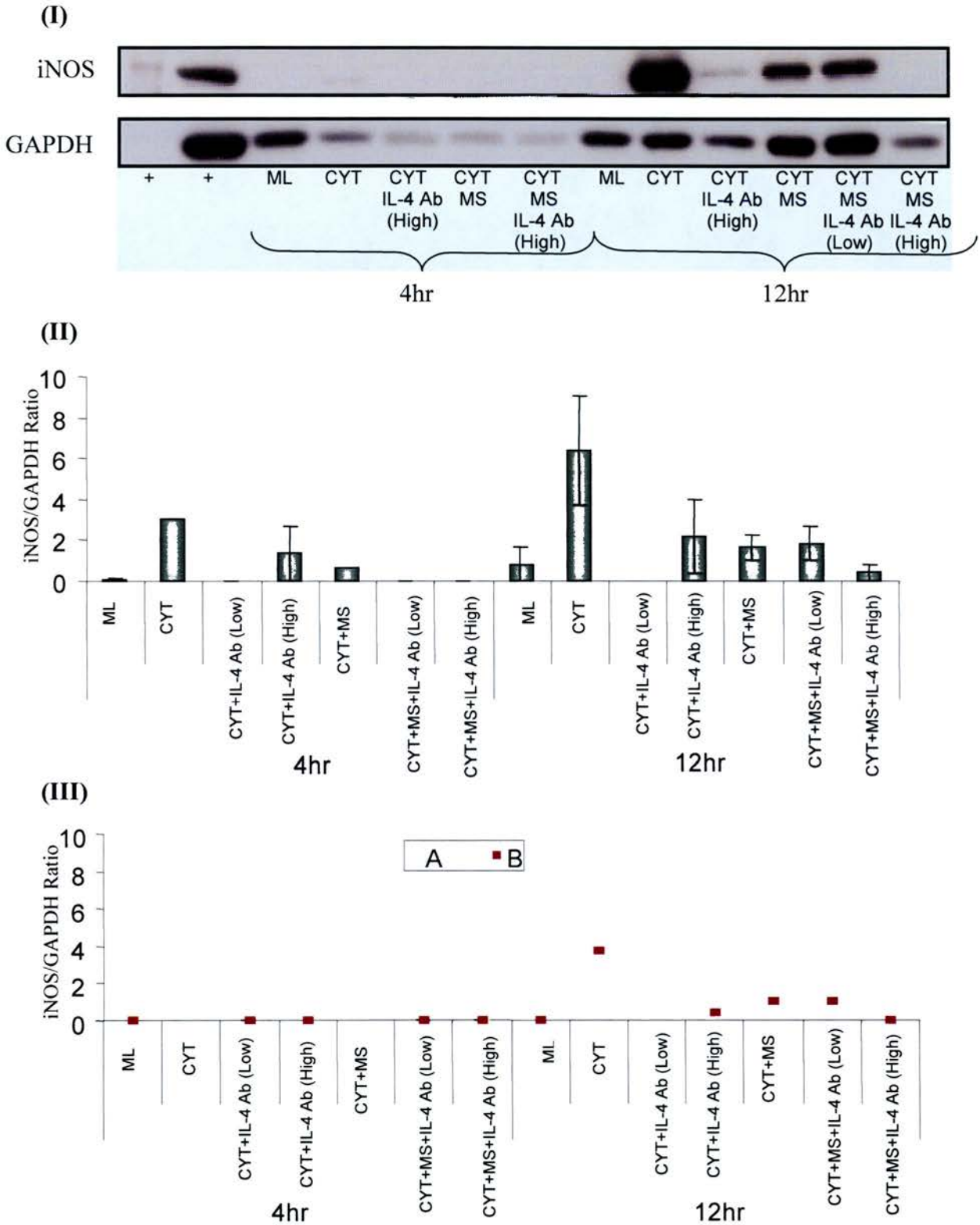


Figure 4.8 – Densitometry data from western blots using polyclonal IL-4 neutralising antibody. (I) = iNOS western blot. (II) = Pooled densitometry values±SEM. (III) = Individual densitometry experimental values. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. IL-4 Ab = Polyclonal IL-4 antibody at 1μg/ml (Low) and 10μg/ml (High). MS = Mechanical stimulation. A=5/9/03 B=3/9/03. (n=2)

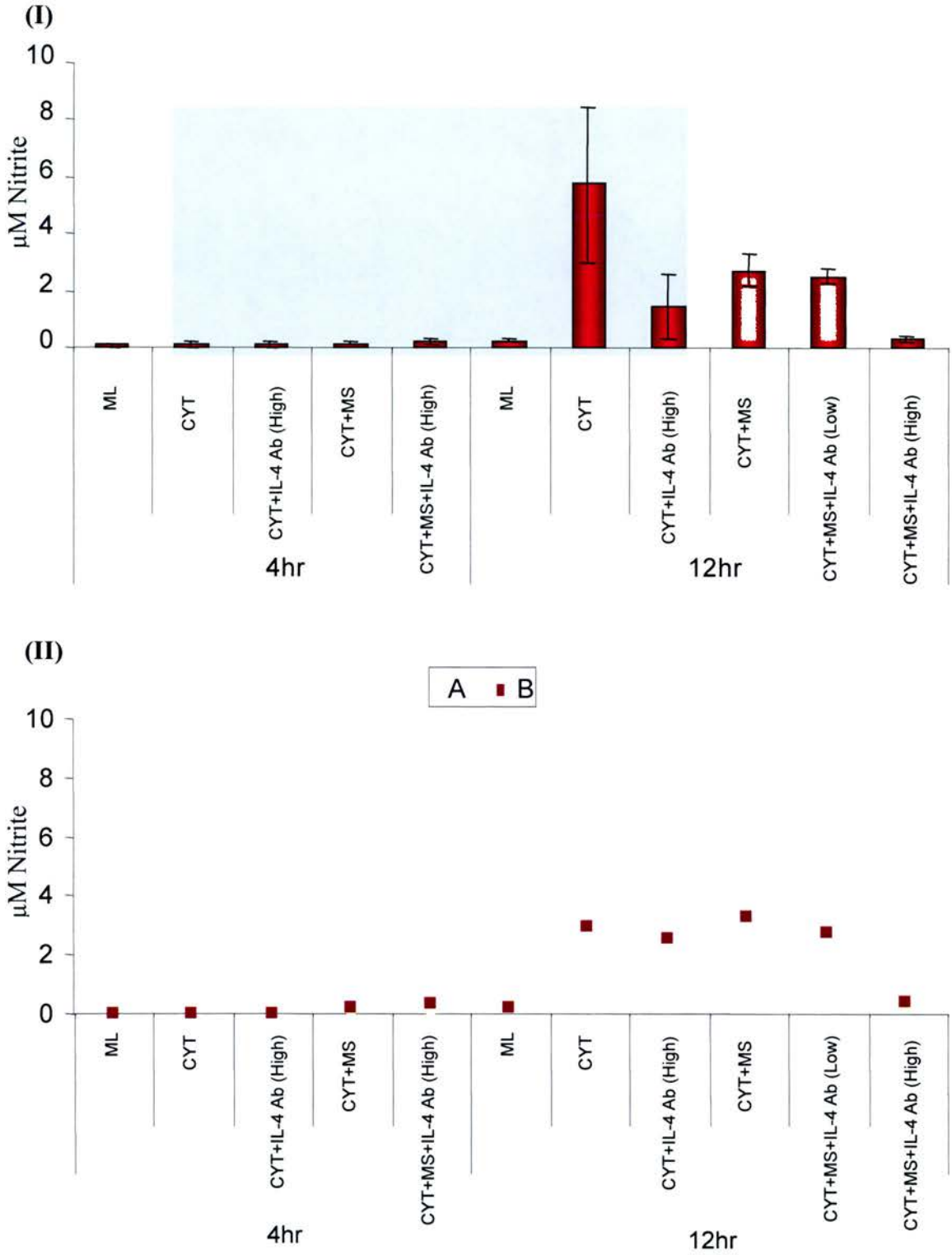


Figure 4.9 – Griess assay nitrite data using polyclonal IL-4 neutralising antibody. (I) = Pooled Griess assay data adjusted for protein levels in the original sample±SEM. (II) = Individual Griess assay experimental data. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. IL-4 Ab = Polyclonal IL-4 neutralising antibody at 1µg/ml (Low) and 10µg/ml (High). MS = Mechanical stimulation. A=5/9/03 B=3/9/03. (n=2)

4.2.3b – Monoclonal IL-4 neutralising Antibody

iNOS mRNA, protein and nitrite levels were measured following CYT stimulation, and the effects of MS and a monoclonal IL-4 neutralising antibody at low (1μg/ml) and high (10μg/ml) concentrations were evaluated.

iNOS mRNA levels were elevated following CYT stimulation for both 4 and 12 hours (**Figure 4.10**). The simultaneous application of CYT and MS also elevated iNOS mRNA levels. However these were 43% lower after 4 hours and 83% lower after 12 hours, than following CYT stimulation alone ($p=0.2$ and $p=0.04$ respectively). The addition of 1μg/ml of a monoclonal IL-4 neutralising antibody did not alter iNOS mRNA levels following stimulation with CYT or CYT plus MS for 4 or 12 hours.

iNOS protein levels were increased by CYT stimulation at both 4 and 12 hours (**Figure 4.11**). Increased iNOS protein was also seen following the simultaneous stimulation with CYT and MS. However these were 88% lower after 4 hours and 83% lower after 12 hours, than following CYT alone ($p<0.05$). The addition of a monoclonal IL-4 neutralising antibody at 1μg/ml or 10μg/ml did not influence iNOS protein levels at 4 or 12 hours in combination with CYT or CYT plus MS.

Nitrite levels were detected at 0.2μM at 4 hours in the medium of cells subsequently extracted for protein (**Figure 4.12**) and mRNA (**Figure 4.13**) under all conditions, which is at the limits of detection for the assay. CYT stimulation increased nitrite levels at 12 hours to 4.2 and 7.3μM for mRNA- and protein-adjusted experiments respectively. CYT plus MS elevated nitrite levels to 3.5 and 1.5μM, 79% lower for protein-adjusted and 17% lower for mRNA-adjusted nitrite than following CYT alone, however these did not reach statistical significance. The presence of the monoclonal IL-4 neutralising antibody, at both 1 and 10μg/ml, had no effect on the level of nitrite detected after cytokine stimulation alone, or following the simultaneous application of CYT and MS.

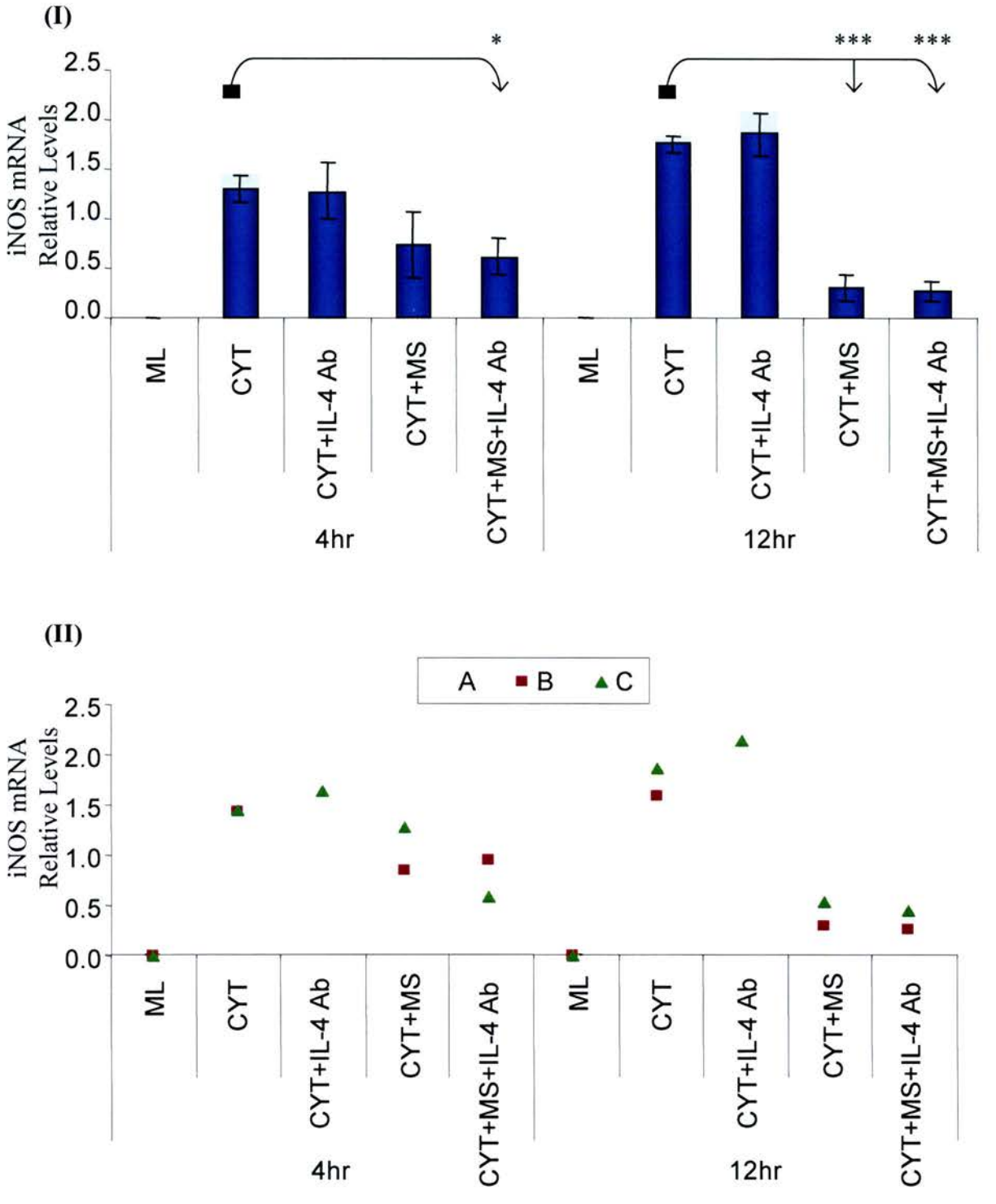


Figure 4.10 – iNOS mRNA levels following incubation with a monoclonal IL-4 neutralising antibody.
(I) = Pooled data±SEM. (II) = Individual experimental values.
ML = Unstimulated control. CYT = Cytokine cocktail stimulation. IL-4 Ab = Monoclonal IL-4 neutralising antibody at 1µg/ml. MS = Mechanical stimulation. A = 3/10/03 B = 18/4/04 C = 29/3/04. (n=3). *$p<0.05$ ***$p<0.001$.

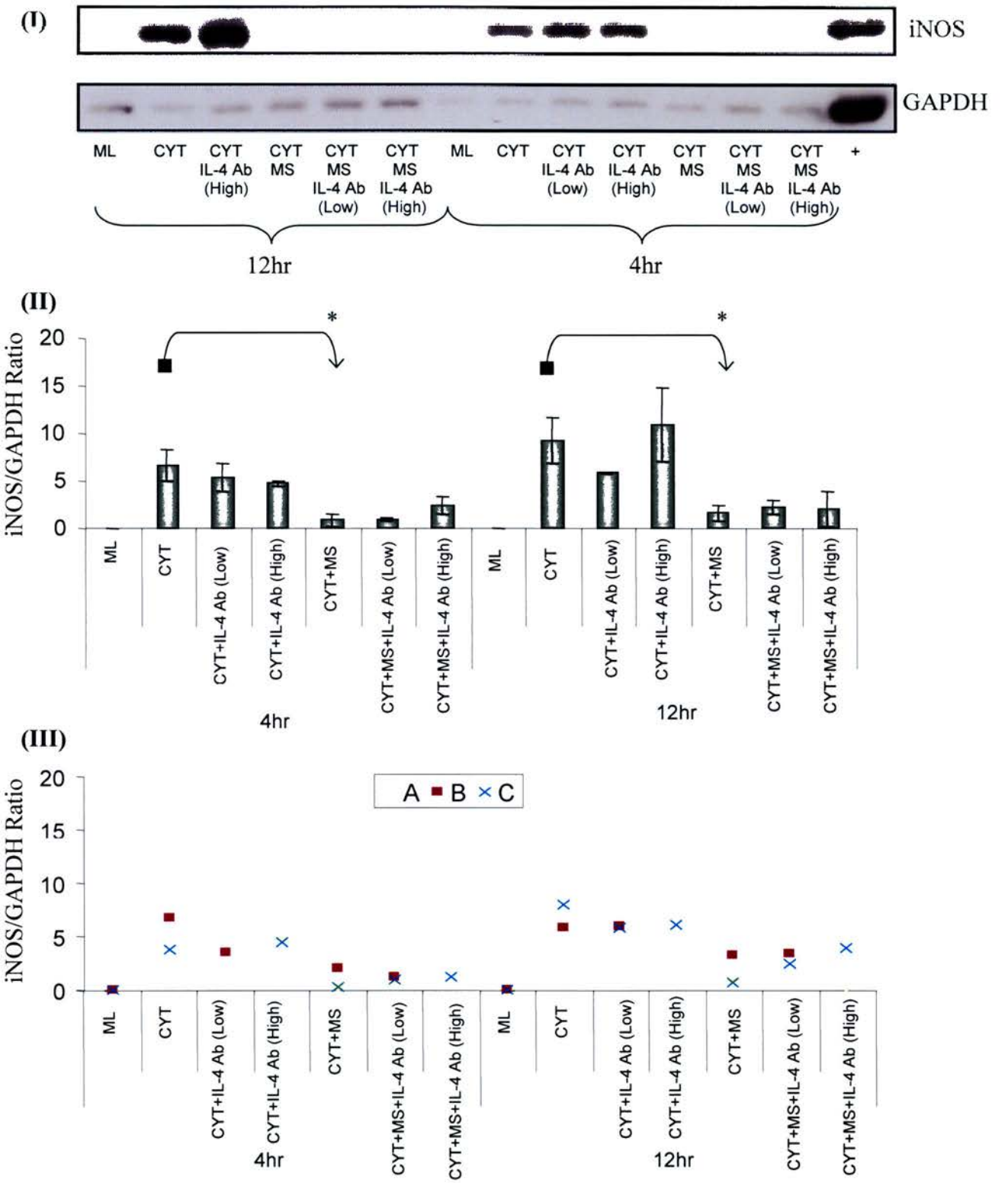


Figure 4.11 – iNOS protein levels following incubation with a monoclonal IL-4 neutralising antibody. (I) = Western blot for iNOS (3/10/03). (II) = Pooled densitometry data from iNOS western blots±SEM. (III) = Individual iNOS densitometry values. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. IL-4 Ab = Monoclonal IL-4 antibody at 1μg/ml (Low) and 10μg/ml (High). MS = Mechanical stimulation. + = CYT stimulated DLD-1 cells. A=3/10/03 B=11/11/03 C=23/9/03. (n=3). *$p<0.05$.

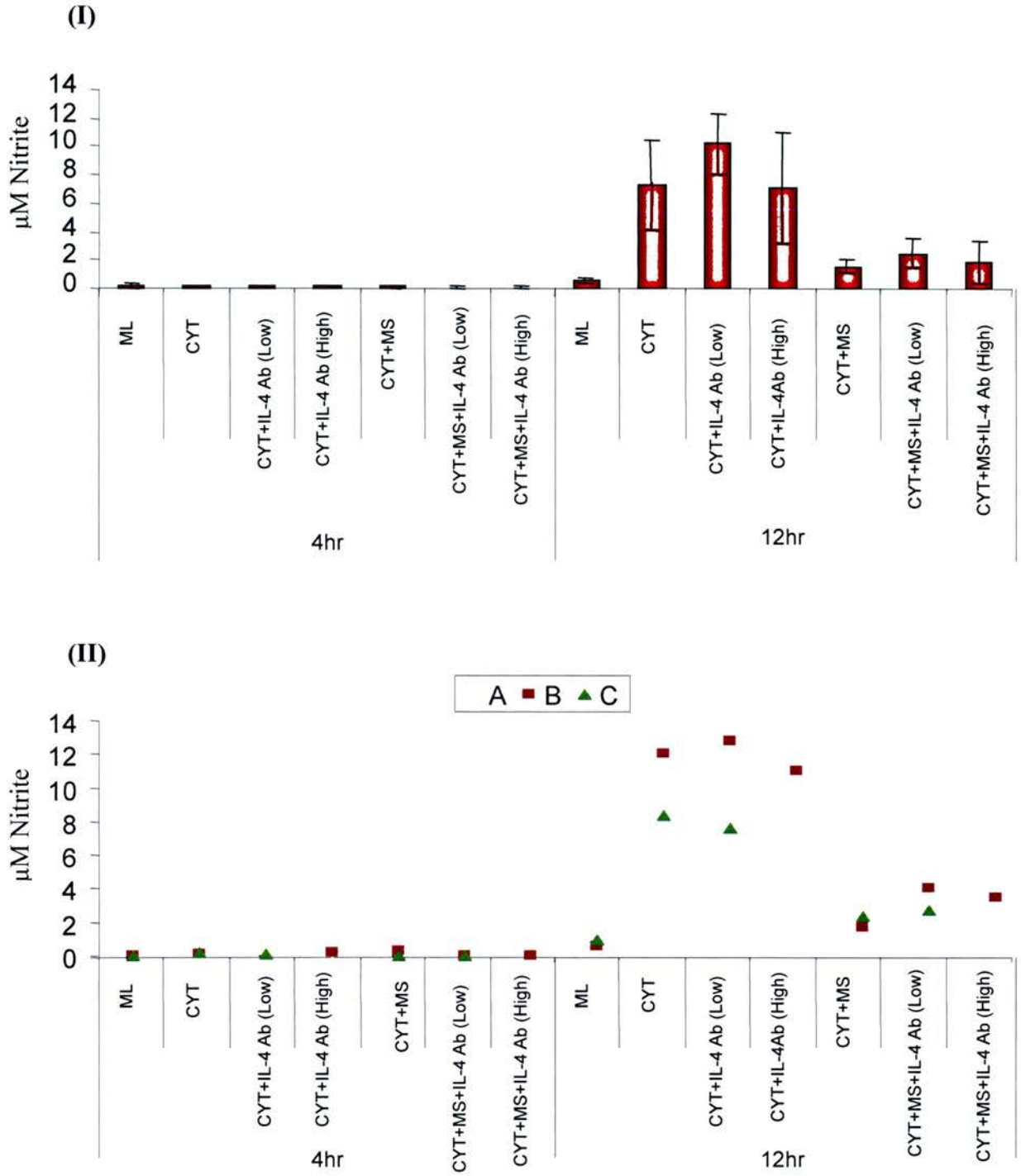


Figure 4.12 – Griess assay protein adjusted nitrite levels following incubation with a monoclonal IL-4 neutralising antibody. (I) = Pooled protein adjusted nitrite values±SEM. (II) = Individual protein adjusted nitrite experimental values. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. IL-4 Ab = Monoclonal IL-4 antibody at 1µg/ml (Low) and 10µg/ml (High). MS = Mechanical stimulation. A=3/10/03 B=23/9/03 C=11/11/03. (n=3)

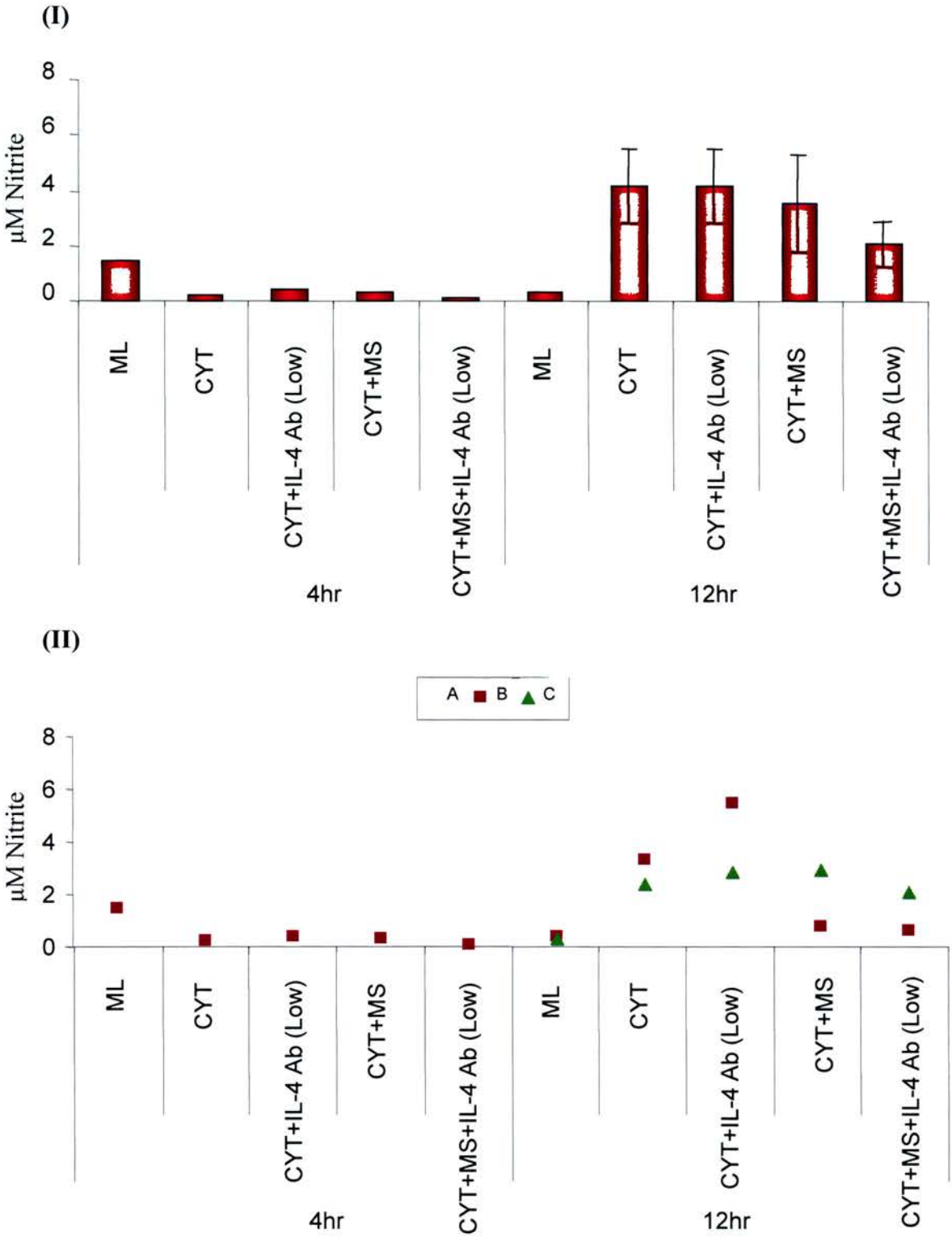


Figure 4.13 - Griess assay mRNA adjusted nitrite levels following incubation with a monoclonal IL-4 neutralising antibody. (I) = Pooled mRNA adjusted nitrite values±SEM. (II) = Individual mRNA adjusted nitrite experimental values. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. IL-4 Ab = Monoclonal IL-4 antibody at 1µg/ml (Low) and 10µg/ml (High). MS = Mechanical stimulation. A=18/4/04 B=3/10/03 C=29/3/04. (n=3)

4.2.4 – Effects of MS and a β1 integrin function blocking antibody on CYT induced iNOS

CYT-induced iNOS mRNA, protein and nitrite levels were measured following MS and incubation with a β1 integrin function blocking antibody (1μg/ml).

iNOS mRNA levels were increased following CYT stimulation at both 4 and 12 hours (**Figure 4.14**). The simultaneous application of CYT and MS also elevated iNOS mRNA, however this was 57% lower after 4 hours and 85% lower after 12 hours, than following CYT alone ($p \leq 0.003$). Incubation with a β1 integrin function blocking antibody at 1μg/ml did not alter the iNOS mRNA levels following CYT or simultaneous CYT and MS.

iNOS protein levels are elevated by CYT at both 4 and 12 hours (**Figure 4.15**). The simultaneous application of CYT and MS also increases iNOS protein above unstimulated controls, however a 66% lower level was seen at 12 hours than following CYT alone ($p=0.08$). Incubation with a β1 integrin function blocking antibody does not alter the iNOS protein level following CYT or simultaneous CYT plus MS.

Nitrite levels were detected below 0.2μM following all stimuli at 4 hours and are at 0.6μM in unstimulated controls at 12 hours, which is at the limits of detection for the assay. CYT stimulation induced the elevation of nitrite levels to 5.8 and 9.7μM for mRNA- and protein-adjusted experiments. The nitrite concentration detected following the simultaneous application of MS and CYT was 3.7μM, 37% lower for mRNA-adjusted and 62% lower for protein-adjusted experiments than following CYT alone ($p \leq 0.12$). Incubation with a β1 integrin function blocking antibody did not alter the nitrite levels in either the mRNA- (**Figure 4.17**) or protein-adjusted (**Figure 4.16**) experiments following CYT, or CYT and MS simultaneously.

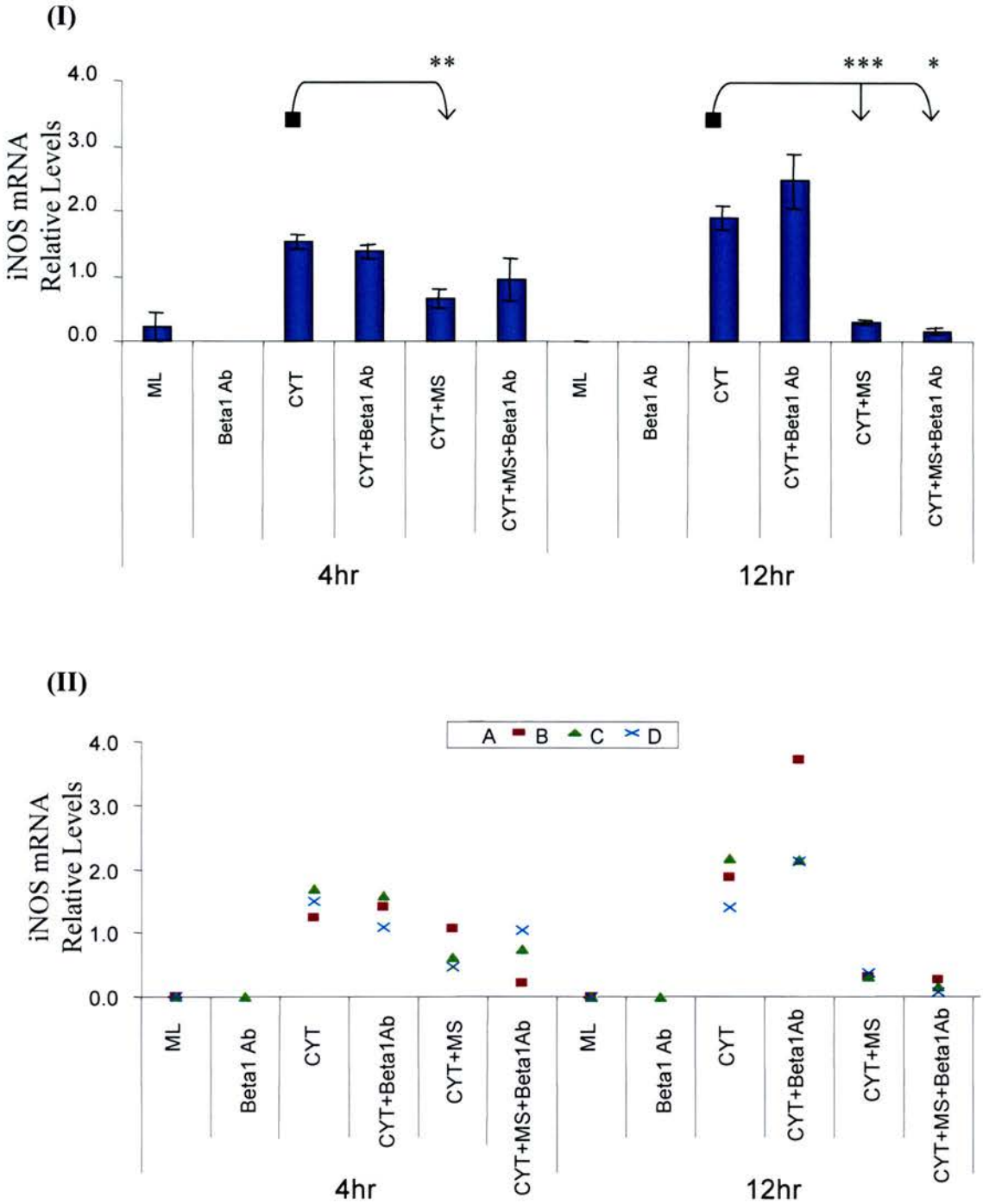
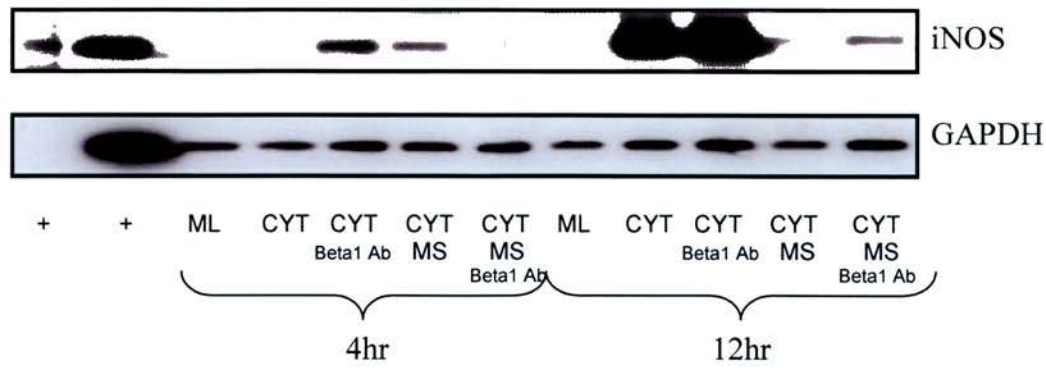


Figure 4.14 - iNOS mRNA levels following incubation with a β1 integrin blocking antibody. (I) = Pooled data±SEM. (II) = Individual experimental values. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. Beta1 Ab = β1 integrin function blocking antibody at 1μg/ml. MS = Mechanical stimulation. A=22/3/04 B=8/3/04 C=10/2/04 D=18/4/04. (n=4). *p<0.05 **p<0.01 ***p<0.001.

(I)



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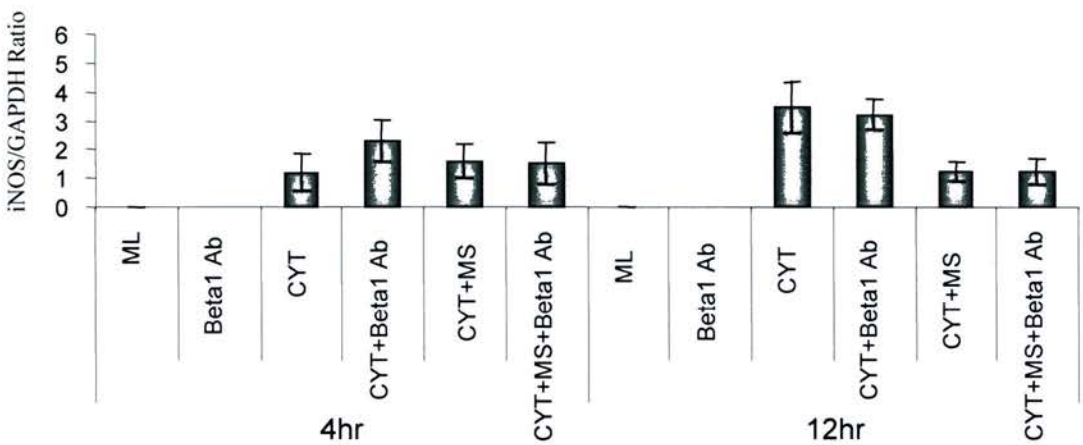

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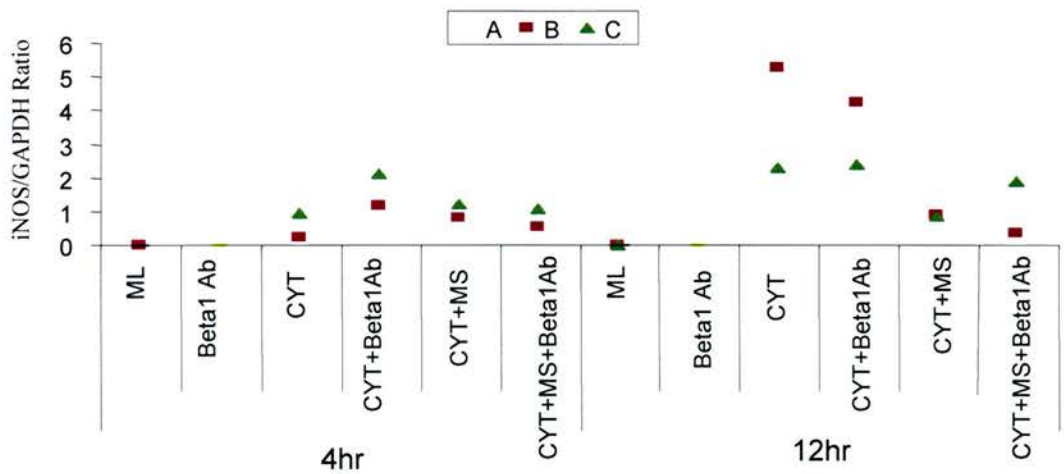


Figure 4.15 - iNOS protein levels following incubation with a β1 integrin function blocking antibody. (I) = Western blot for iNOS (14/2/04). (II) = Pooled densitometry data from iNOS western blots±SEM. (III) = Individual iNOS densitometry values. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. Beta1 Ab = Beta1 integrin function blocking antibody at 1μg/ml. MS = Mechanical stimulation. A=10/2/04 B=14/2/04 C=22/3/04. (n=3)

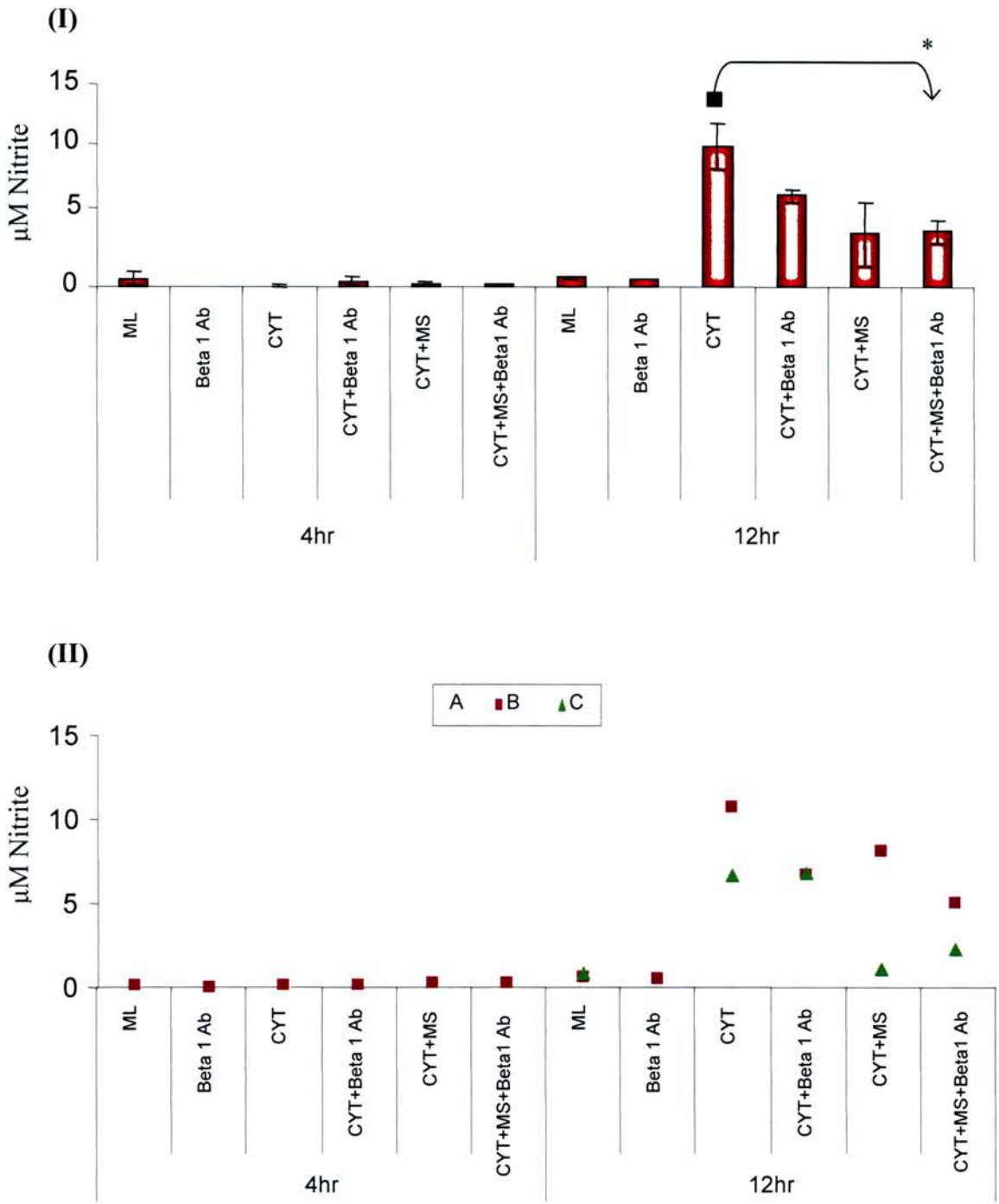


Figure 4.16 - Griess assay protein adjusted nitrite levels following incubation with a β1 integrin function blocking antibody. (I) = Pooled protein adjusted nitrite values±SEM. (II) = Individual protein adjusted nitrite experimental values. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. Beta1 Ab = β1 integrin function blocking antibody at 1µg/ml. MS = Mechanical stimulation. A=22/3/04 B=10/2/04 C=14/2/04. (n=3). *$p<0.05$.

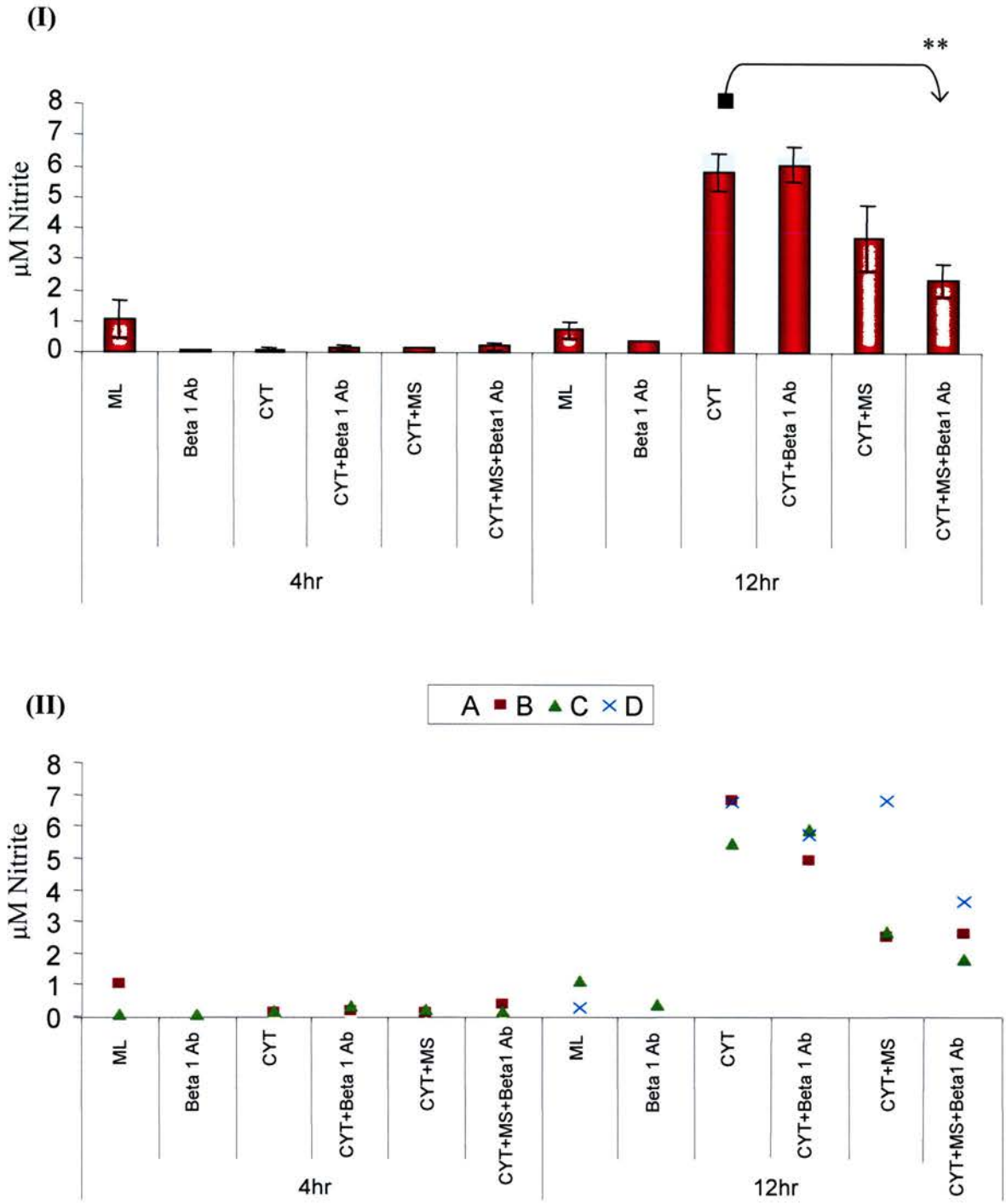


Figure 4.17 - Griess assay mRNA adjusted nitrite levels following incubation with a β1 integrin function blocking antibody. (I) = Pooled mRNA adjusted nitrite values±SEM. (II) = Individual mRNA adjusted nitrite experimental values. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. Beta1 Ab = β1 integrin function blocking antibody at 1µg/ml. MS = Mechanical stimulation. A=22/3/04 B=8/3/04 C=10/2/04 D=18/4/04. (n=4). **p<0.01.

4.2.5 – Effects of MS, IL-4 neutralising antibody and β1 integrin function blocking antibody on CYT induced iNOS – pooled data

iNOS mRNA levels were elevated following CYT stimulation for 4 and 12 hours (**Figure 4.18**). The simultaneous application of CYT and MS also elevated iNOS mRNA levels. However these were 55% lower at 4 hours and 86% lower at 12 hours, than following CYT alone ($p<0.001$). Incubation with both a monoclonal IL-4 neutralising antibody and a β1 integrin function blocking antibody (both 1μg/ml) had no significant effect on iNOS mRNA levels following CYT plus MS. The IL-4 antibody had no effect on iNOS mRNA levels following CYT, while the β1 antibody increased the levels of iNOS following CYT with this increase almost reaching significance ($p=0.051$).

iNOS protein levels were elevated by CYT at both 4 and 12 hours from the undetectable levels in the unstimulated control (**Figure 4.19**). The simultaneous application of CYT and MS also elevated iNOS protein levels, although this was 70% lower at 4 hours and 76% lower at 12 hours than following CYT alone ($p=0.04$ and $p=0.0003$ respectively). Incubation with either a monoclonal IL-4 neutralising antibody or a β1 integrin function blocking antibody (both 1μg/ml) showed no significant alteration of the iNOS protein level following CYT or simultaneous CYT and MS.

Nitrite levels were detected below 0.5μM at 4 hours following all stimuli and at 12 hours in the unstimulated control, which is at the limits of detection for the assay. Following 12 hours CYT nitrite levels were elevated to 6 and 7.5μM in mRNA (**Figure 4.20**) and protein (**Figure 4.21**) adjusted experiments respectively. The simultaneous application of MS and CYT also increased nitrite at 12 hours, however this was 58% lower for mRNA-adjusted and 63% lower for protein-adjusted than following CYT alone ($p<0.001$). Incubation with either a monoclonal IL-4 neutralising antibody or a β1 integrin function blocking antibody (both 1μg/ml) showed no significant alteration of the nitrite levels following CYT, or simultaneous CYT and MS.

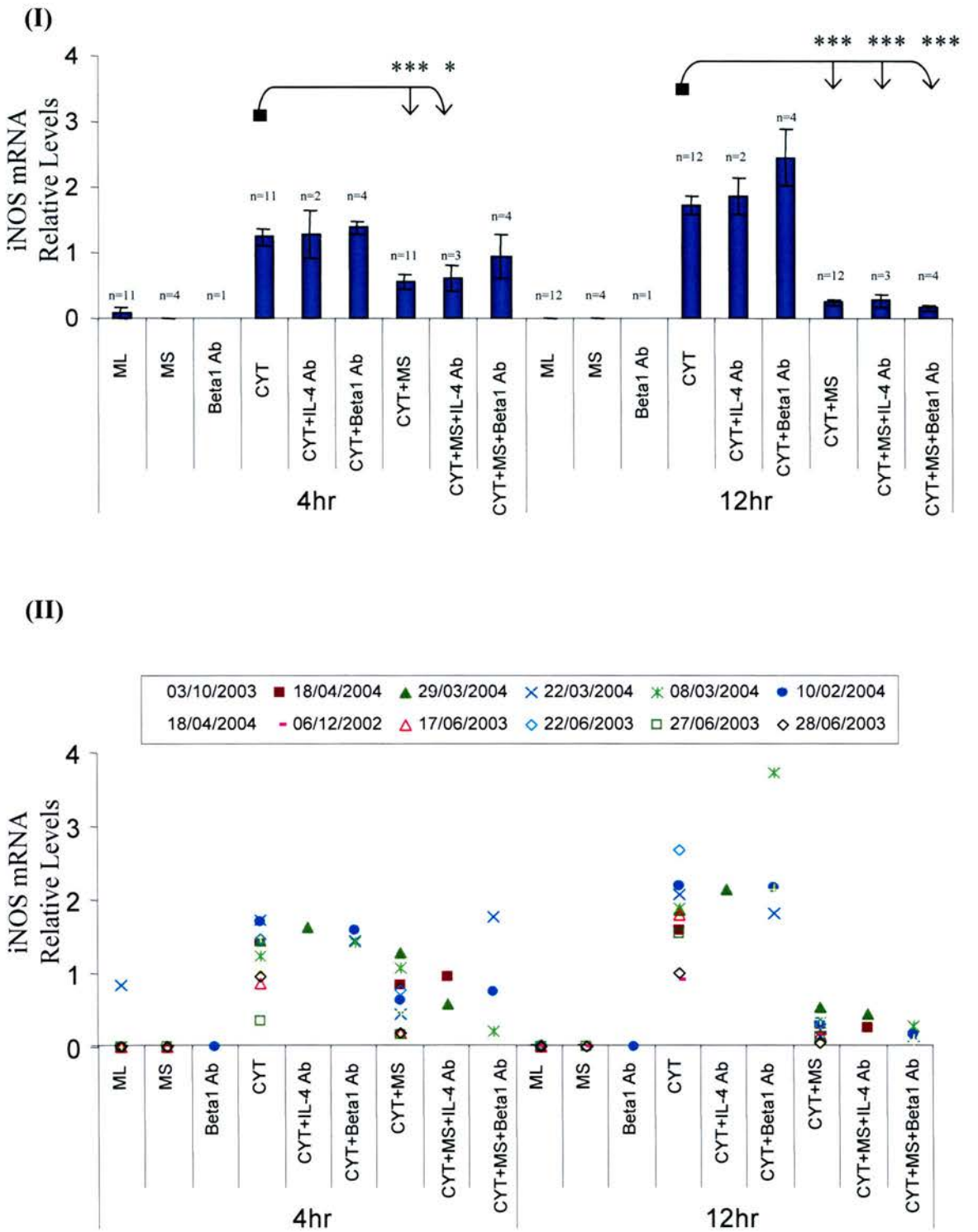


Figure 4.18 – Pooled iNOS mRNA levels following incubation with an IL-4 neutralising antibody or a β1 integrin function blocking antibody.
(I) =Pooled data±SEM. (II) = Individual experimental values.
ML = Unstimulated control. CYT = Cytokine cocktail stimulation.
Beta1 Ab = β1 integrin function blocking antibody at 1μg/ml.
IL-4 Ab = Monoclonal IL-4 neutralising antibody at 1μg/ml.
MS = Mechanical stimulation. *$p<0.05$ ***$p<0.001$.

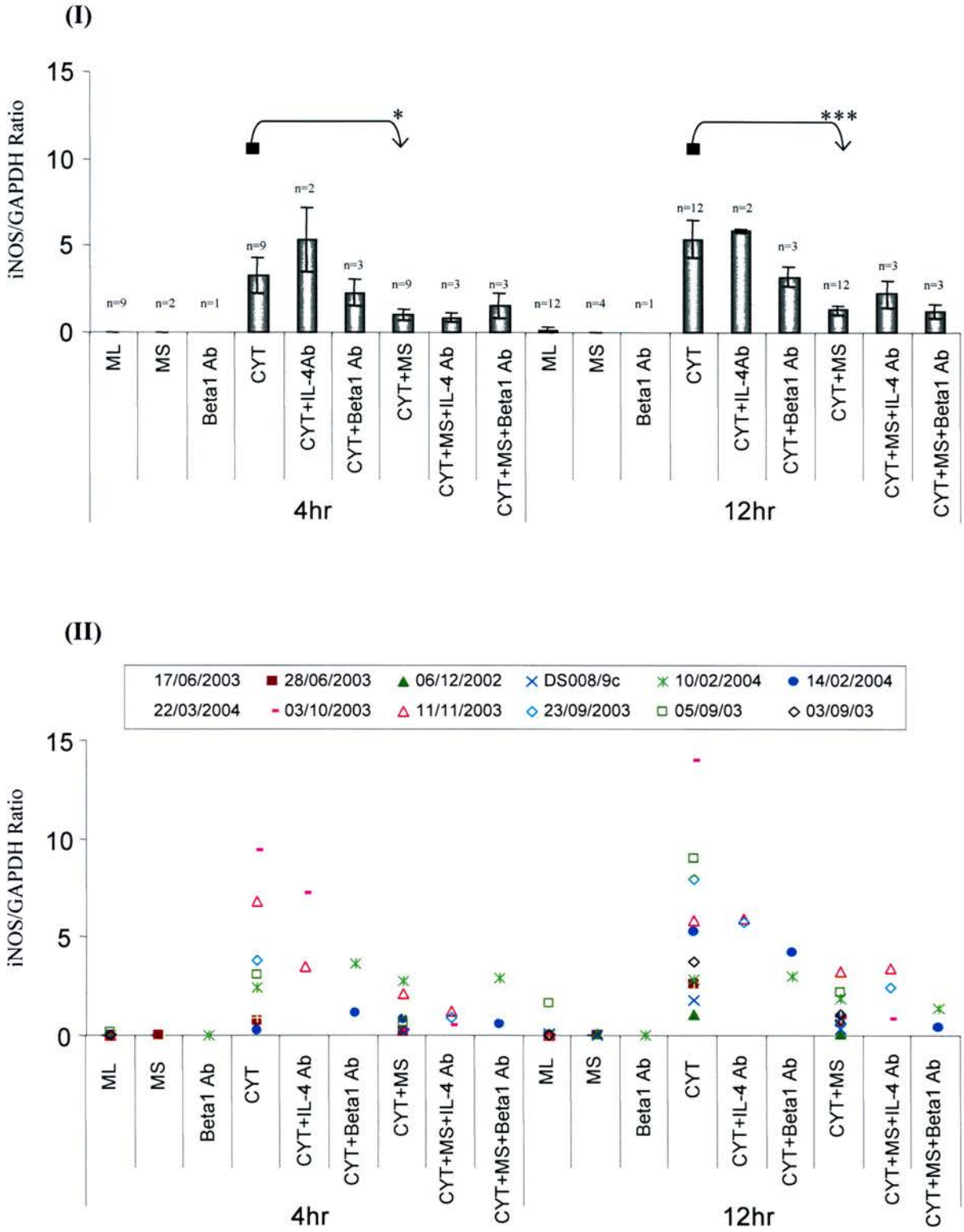


Figure 4.19 – Pooled iNOS protein levels following incubation with an IL-4 neutralising antibody or a β1 integrin function blocking antibody. (I) =Pooled data±SEM. (II) = Individual experimental values. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. Beta1 Ab = β1 integrin function blocking antibody at 1μg/ml. IL-4 Ab = Monoclonal IL-4 neutralising antibody at 1μg/ml. MS = Mechanical stimulation. *$p<0.05$ ***$p<0.001$.

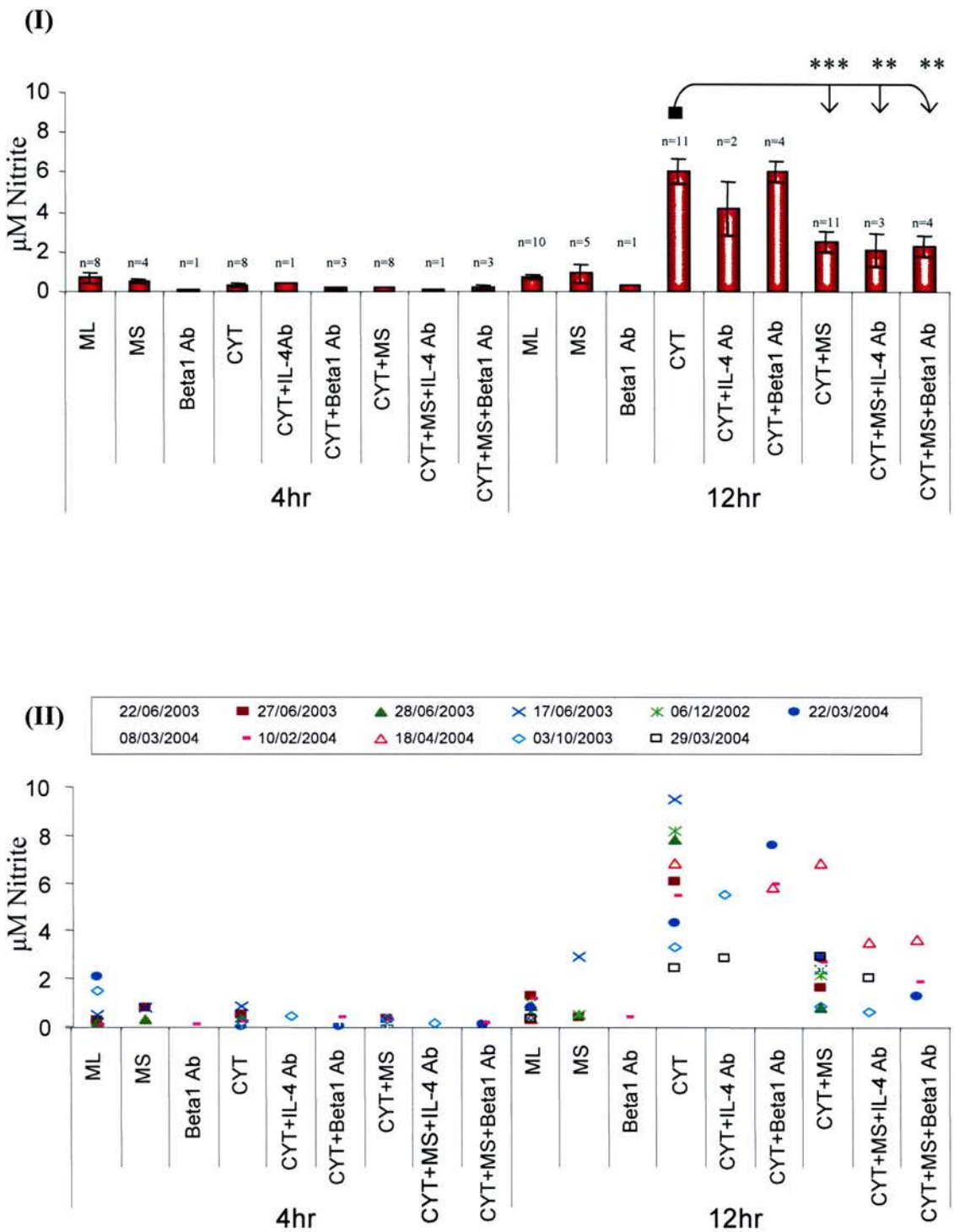


Figure 4.20 – Pooled mRNA adjusted nitrite levels following incubation with an IL-4 neutralising antibody or a β1 integrin function blocking antibody. (I) =Pooled mRNA adjusted data±SEM. (II) = Individual experimental values. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. Beta1 Ab = β1 integrin function blocking antibody at 1μg/ml. IL-4 Ab = Monoclonal IL-4 neutralising antibody at 1μg/ml. MS = Mechanical stimulation. **p<0.01 ***p<0.001.

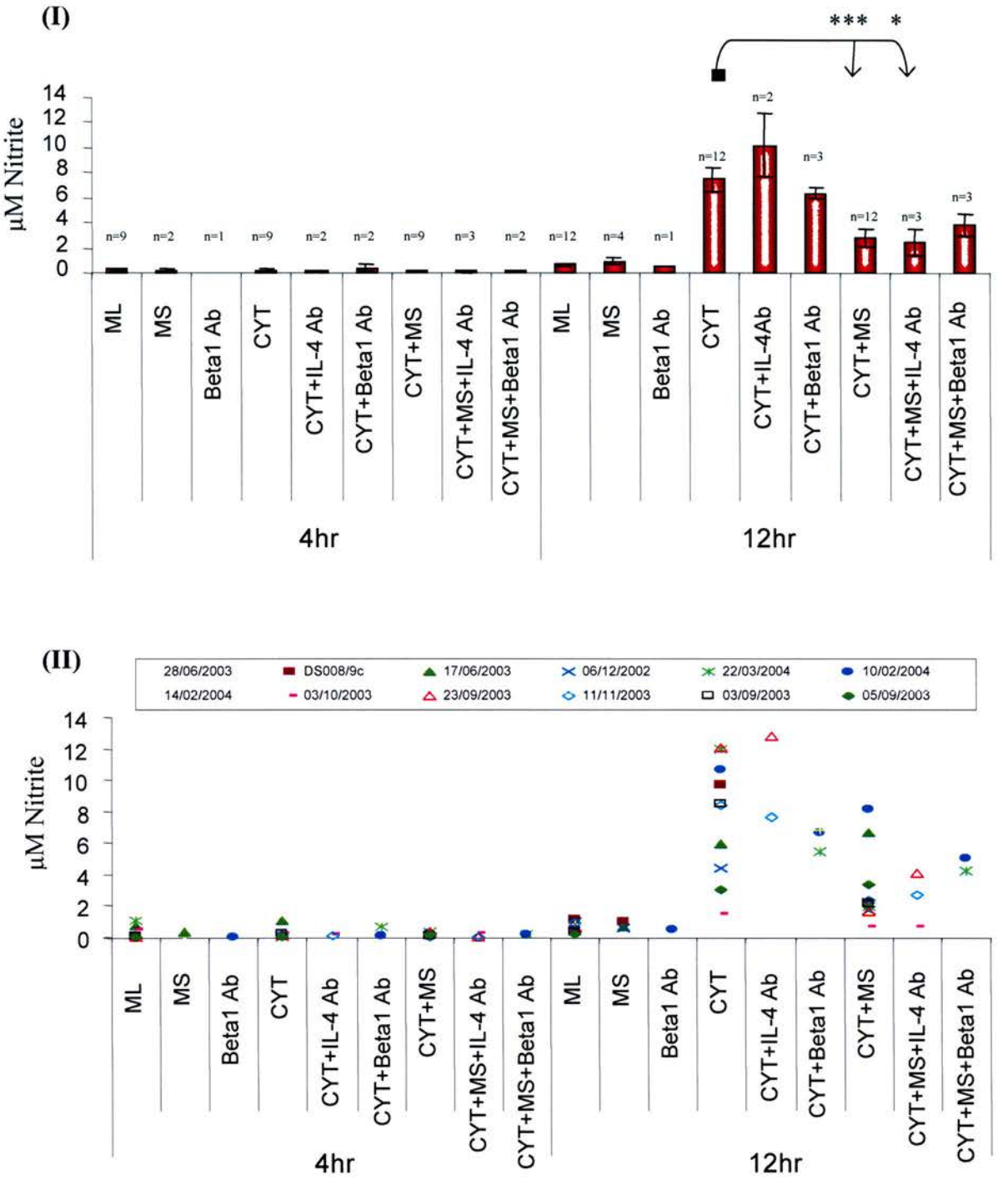


Figure 4.21 – Pooled protein adjusted nitrite levels following incubation with an IL-4 neutralising antibody or a β1 integrin function blocking antibody.
(I) =Pooled protein adjusted data±SEM. (II) = Individual experimental values.
ML = Unstimulated control. CYT = Cytokine cocktail stimulation.
Beta1 Ab = β1 integrin function blocking antibody at 1μg/ml.
IL-4 Ab = Monoclonal IL-4 neutralising antibody at 1μg/ml.
MS = Mechanical stimulation. *$p<0.05$ ***$p<0.001$.

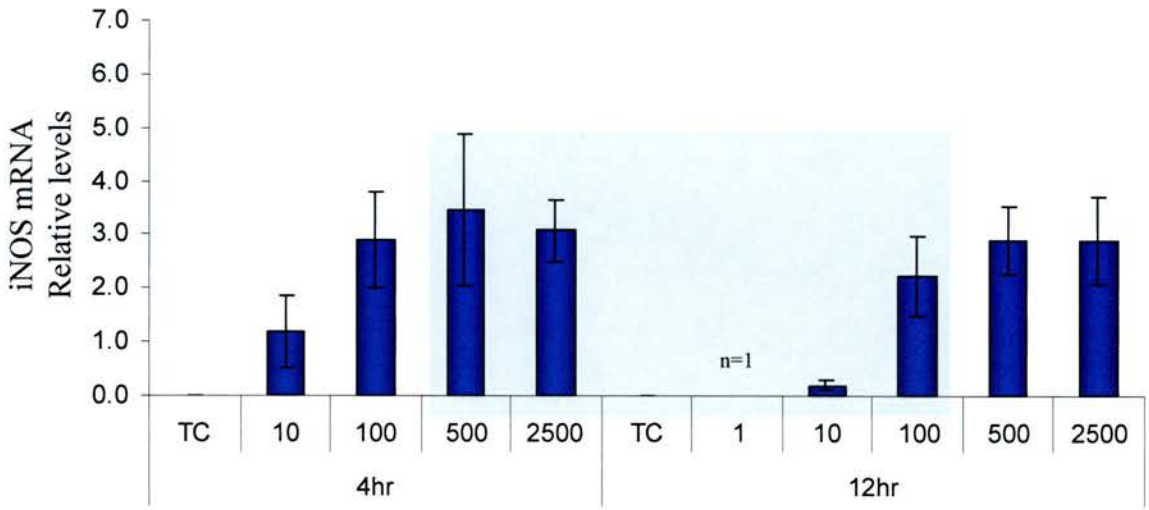
4.2.6 – Effects of IL-1β at a range of concentrations on iNOS mRNA, protein and nitrite levels

iNOS mRNA levels were elevated from unstimulated controls at IL-1β concentrations ranging from 10pg/ml to 2500pg/ml ($p=0.1$) (**Figure 4.22**). 10pg/ml IL-1 elevated iNOS mRNA levels by 397 and 96.5 fold above unstimulated controls at 4 and 12 hours respectively. 100pg/ml elevated iNOS mRNA levels above 10pg/ml by 2.4 fold at 4 and 12.6 fold at 12 hours. 500pg/ml did not increase iNOS mRNA levels above 100pg/ml at 4 hours, however a 1.3 fold increase was seen at 12 hours. 2500pg/ml did not elevate iNOS mRNA levels above 500pg/ml at 4 or 12 hours.

iNOS protein was detected following 4 hours IL-1β, and at higher levels following 12 hours IL-1β stimulation at concentrations ranging from 25pg/ml to 10000pg/ml (**Figure 4.23**). Primary HAC stimulated with 25pg/ml IL-1β showed elevated iNOS protein compared to the unstimulated control at both 4 and 12 hours. 100 and 10000pg/ml IL-1β showed similar levels of iNOS protein, 2.1 fold higher at 4 hours and 1.5 fold higher at 12 hours than following 25pg/ml stimulation. 500pg/ml IL-1β at 4 hours showed an iNOS protein level similar to that seen for 25pg/ml, while at 12 hours they were 1.5 fold higher than 25pg/ml. 2500pg/ml IL-1β at 4 hours had a 2.4 fold higher iNOS protein level than 25pg/ml, while at 12 hours they were similar to that seen following 25pg/ml stimulation. This experiment was not repeated, so statistical analysis can not be performed.

Nitrite levels were below 0.7μM at 4 hours for all IL-1β concentrations investigated, and in the unstimulated control at 12 hours, which is at the limits of detection for the assay. At 12 hours the nitrite levels were elevated to 2.3μM following 10pg/ml and 6.7μM following 2500pg/ml IL-1β stimulation (**Figures 4.23 and 4.24**). Nitrite levels were elevated 4.6 fold following 10pg/ml IL-1β compared to the unstimulated control but this increase was not significant. The nitrite levels detected following 100, 500 and 2500pg/ml showed significant increases compared to the unstimulated control ($p<0.01$). 100pg/ml IL-1β showed a 2.2 fold elevation above that following 10pg/ml IL-1β. 500pg/ml IL-1β showed a further 1.4 fold elevation in nitrite levels. 2500pg/ml IL-1β did not alter nitrite levels compared to those seen following 500pg/ml in the mRNA adjusted experiment (**Figure 4.24**). The protein adjusted experiment, carried out on a single occasion, suggested that 2500pg/ml elevates nitrite levels 1.2 fold more than 500pg/ml, with a further increase of 1.2 fold following 10000pg/ml (**Figure 4.23**).

(I)



(II)

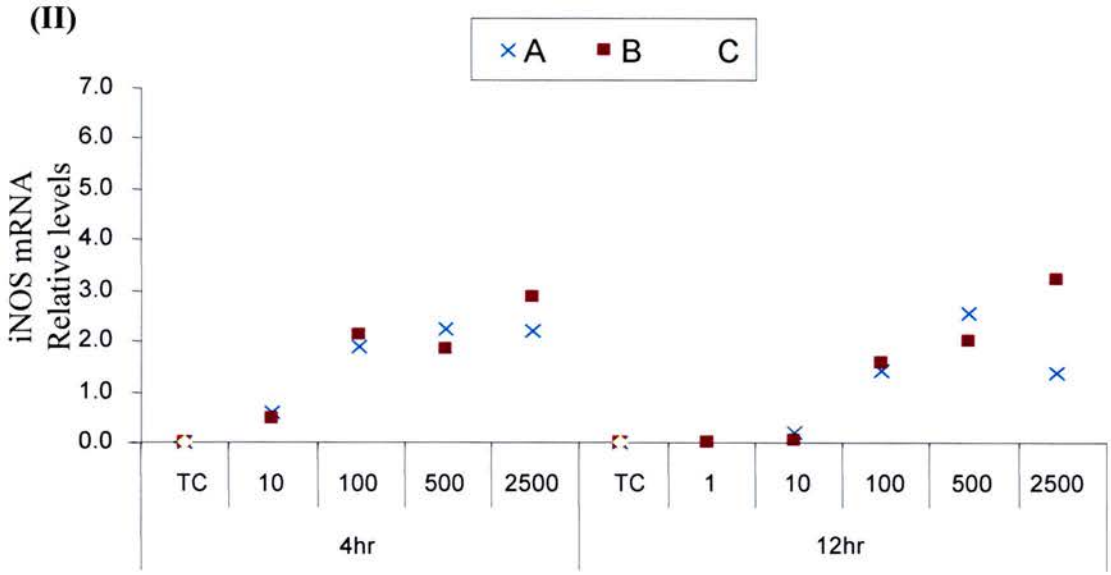
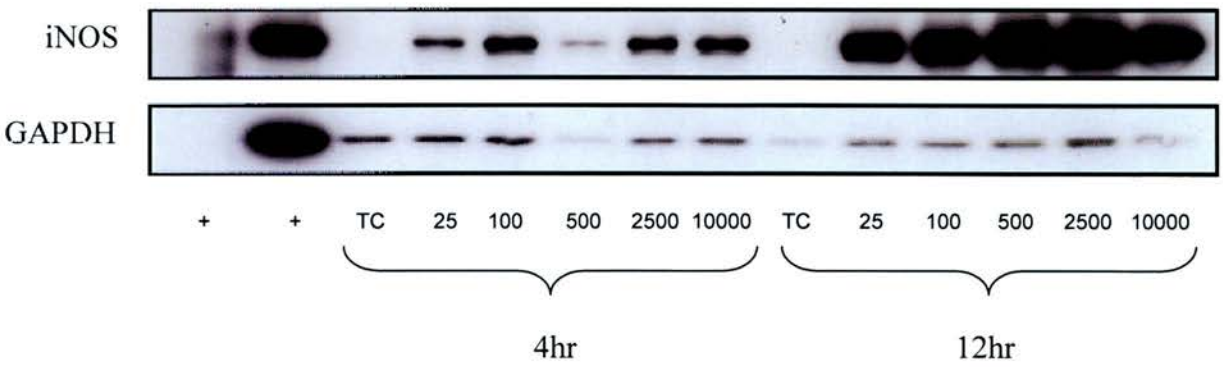
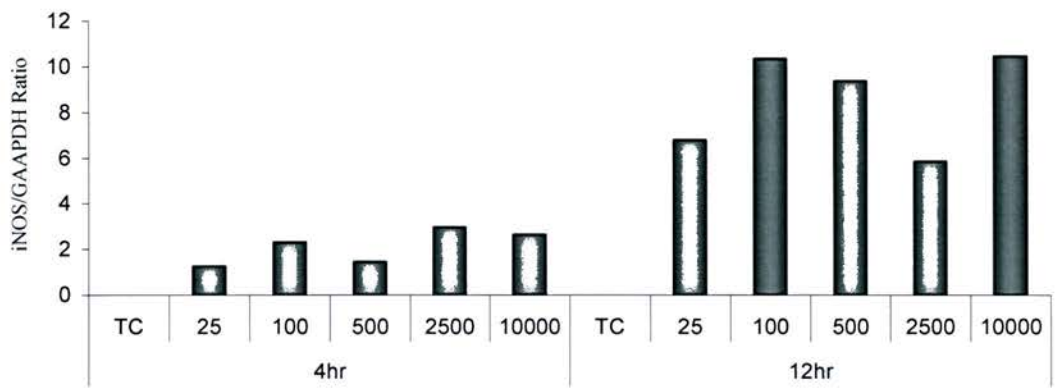


Figure 4.22 - iNOS mRNA levels after IL-1β stimulation at a range of concentrations. IL-1β values in pg/ml. (I) = Pooled data±SEM. (II) = Individual experimental values. TC = Unstimulated control. A=21/5/04 B=16/4/04 C=18/4/04. (n=3)

(I)



(II)



(III)

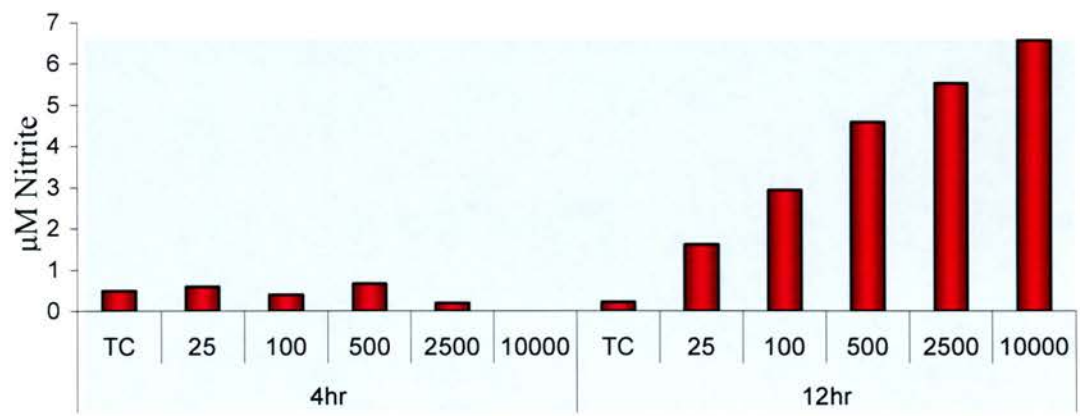


Figure 4.23 – iNOS protein and nitrite after IL-1β stimulation at a range of concentrations. IL-1β values in pg/ml.
(I) = Western blot. (II) = Densitometry Values. (III) = Protein adjusted nitrite values.
TC= Unstimulated control. Experiment number = 21/11/03. (n=1)

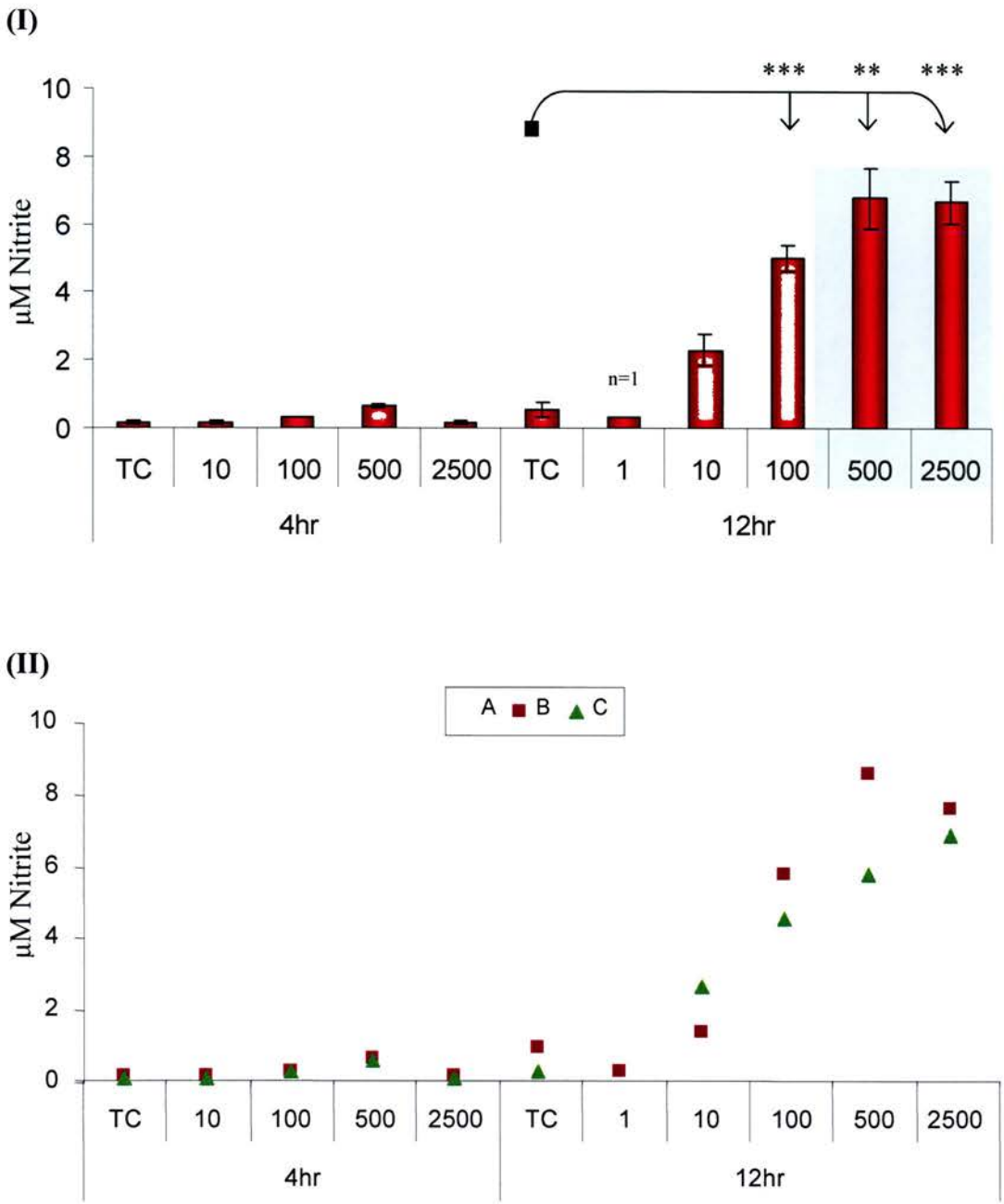


Figure 4.24 – mRNA adjusted nitrite levels after IL-1β stimulation at a range of concentrations. IL-1β values in pg/ml.
(I) = Pooled mRNA adjusted nitrite values±SEM. (II) = Individual mRNA adjusted nitrite experimental values. TC = Unstimulated control. A=21/5/04 B=16/5/04 C=18/5/04. (n=3). **p<0.01 ***p<0.001.

4.2.7 – Effects MS, IL-4 neutralising antibody and β1 integrin function blocking antibody on IL-1β induced iNOS mRNA, protein and nitrite Levels

IL-1β stimulation (500pg/ml) elevated iNOS mRNA levels above unstimulated controls at both 4 and 12 hours (**Figure 4.25**). The simultaneous application of IL-1β and MS also elevated iNOS mRNA levels. However these were 47% lower at 4 hours and 91% lower at 12 hours than following CYT alone ($p=0.28$ and $p=0.1$). The incubation with either a monoclonal IL-4 neutralising antibody or a β1 integrin function blocking antibody (both at 1μg/ml) had no effect on the iNOS mRNA levels following IL-1β alone, or in combination with MS. The exception was IL-1+MS+β1Ab at 4 and 12 hours, where a 50% decrease below IL-1+MS levels was seen, but this was not statistically significant.

Western blotting revealed elevated iNOS protein levels above unstimulated controls following IL-1β stimulation at both 4 and 12 hours (**Figure 4.26 and 4.27**). The simultaneous application of IL-1β plus MS also elevated the iNOS protein level. However this was 72% lower at 4 hours and 23% lower at 12 hours than following IL-1β alone. The densitometry data, however, suggested the difference between cells stimulated with IL-1β alone or in combination with MS was not significant. Incubation with either the monoclonal IL-4 neutralising antibody or β1 integrin function blocking antibody (both at 1μg/ml) had no effect on iNOS protein levels after IL-1β stimulation alone, or in combination with MS.

Nitrite accumulation in the medium was below 0.6μM at 4 hours following all stimuli and in the unstimulated control at 12 hours, which is at the limits of detection for the assay. Following 12 hours of IL-1β stimulation the nitrite concentrations were raised to 6.4 and 12.4μM for mRNA- and protein-adjusted experiments respectively (**Figures 4.28 and 4.29**). The simultaneous application of IL-1β and MS also increased nitrite levels. However these were 41% lower in mRNA adjusted and 23% lower in protein adjusted nitrite values than following IL-1β alone ($p=0.13$ and $p<0.01$ respectively). Incubation with a monoclonal IL-4 neutralising antibody or β1 integrin function blocking antibody (both at 1μg/ml) had no effect on nitrite levels following IL-1β stimulation alone, or in combination with MS. The exception to this was where nitrite levels were reduced 44% following IL-1+MS+β1Ab (mRNA-adjusted) and 9% following IL-1+β1Ab (protein-adjusted) ($p=0.1$).

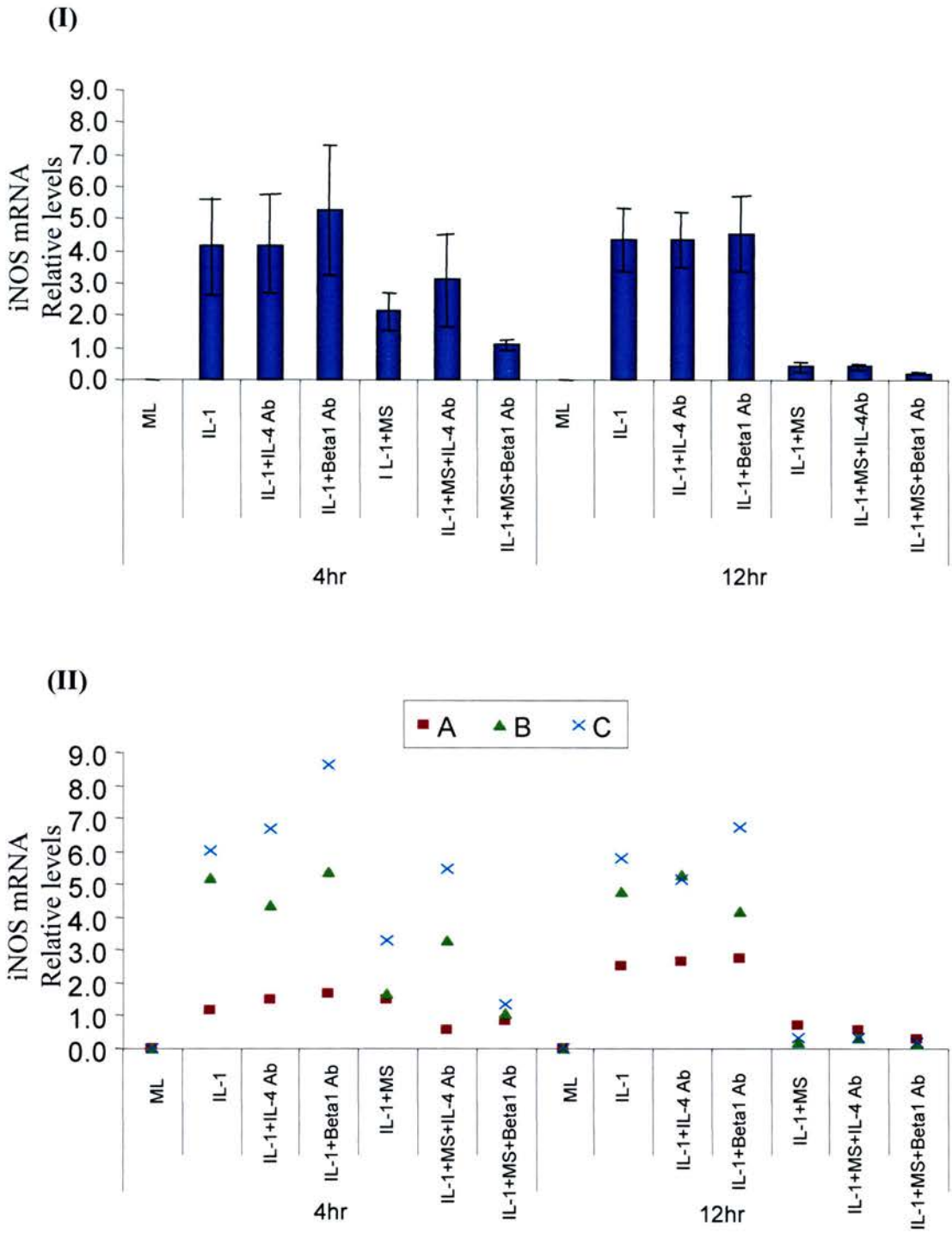


Figure 4.25 - iNOS mRNA levels following incubation with IL-1β, β1 integrin function blocking antibody, monoclonal IL-4 neutralising antibody and MS. (I) = Pooled data±SEM. (II) = Individual experimental values. ML = Unstimulated control. IL-1 = IL-1β stimulation (500pg/ml). Beta1 Ab = β1 integrin blocking antibody (1μg/ml). IL-4 Ab = IL-4 neutralising antibody (1μg/ml). MS = Mechanical stimulation. A=18/4/04 B=6/4/04 C=9/4/04. (n=3)

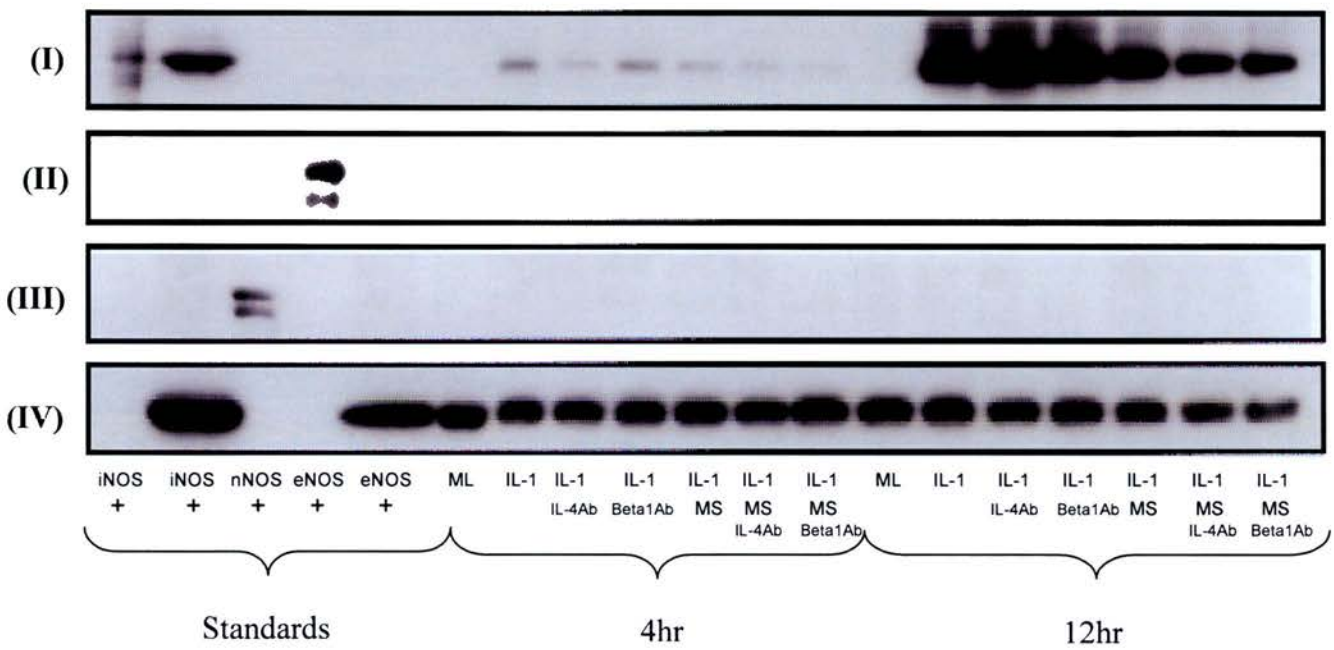


Figure 4.26 – Western blot for NOS isoforms following incubation with IL-1β, MS, β1 integrin function blocking antibody and monoclonal IL-4 neutralising antibody. (I) = iNOS. (II) = eNOS. (III) = nNOS. (IV) = GAPDH. 18/4/04. ML = Unstimulated control. IL-1 = IL-1β stimulation (500pg/ml). Beta1 Ab = β1 integrin function blocking antibody (1μg/ml). IL-4 Ab = IL-4 neutralising antibody (1μg/ml). MS = Mechanical stimulation.

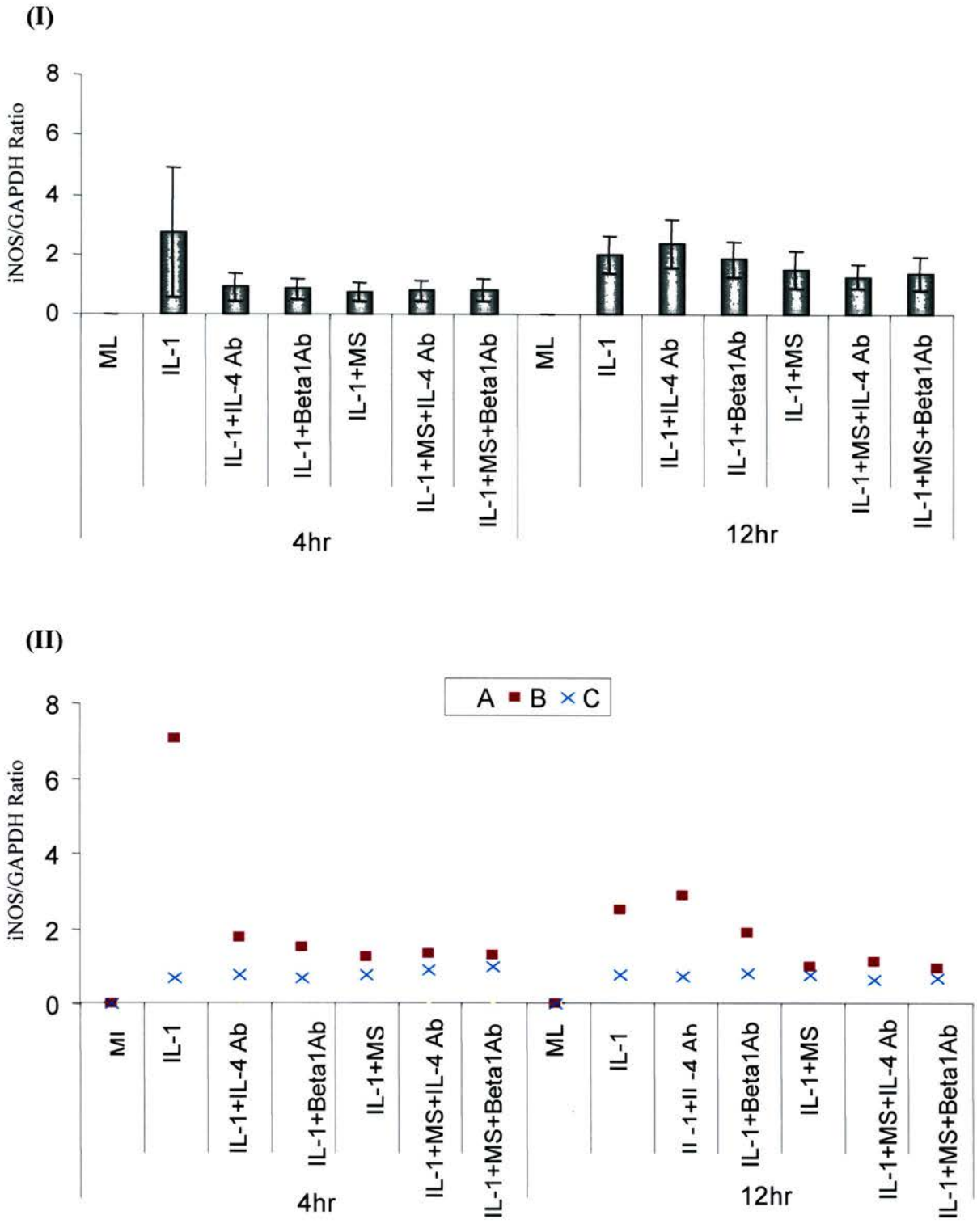


Figure 4.27 – Densitometry data from western blots following IL-1β, β1 integrin function blocking antibody and monoclonal IL-4 neutralising antibody.
(I) = Pooled data±SEM. (II) = Individual experimental values.
ML = Unstimulated control. IL-1 = IL-1β stimulation (500pg/ml). Beta1 Ab = β1 integrin function blocking antibody (1µg/ml). IL-4 Ab = IL-4 neutralising antibody (1µg/ml). MS = Mechanical stimulation. A=18/4/04 B=6/4/04 C=9/4/04. (n=3)

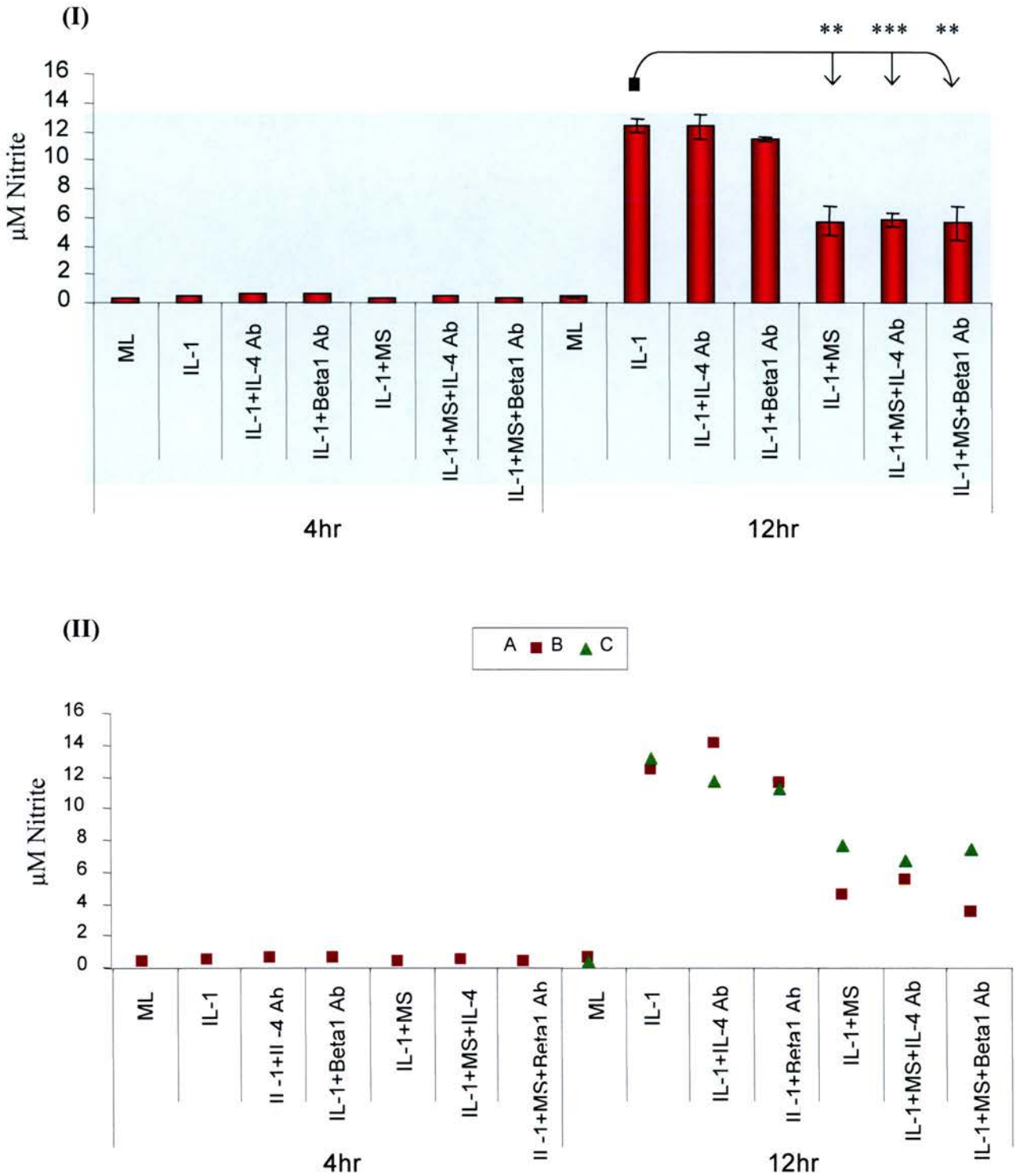


Figure 4.28 – Protein adjusted nitrite data from Griess assay following incubation with IL-1β, β1 integrin blocking antibody and monoclonal IL-4 neutralising antibody. (I) = Pooled data±SEM. (II) = Individual experimental values.
ML = Unstimulated control. IL-1 = IL-1β stimulation (500pg/ml). Beta1 Ab = β1 integrin blocking antibody (1µg/ml). IL-4 Ab = IL-4 neutralising antibody (1µg/ml). MS = Mechanical stimulation. A=6/4/04 B=9/4/04 C=18/4/04. (n=3). **p<0.01 ***p<0.001.

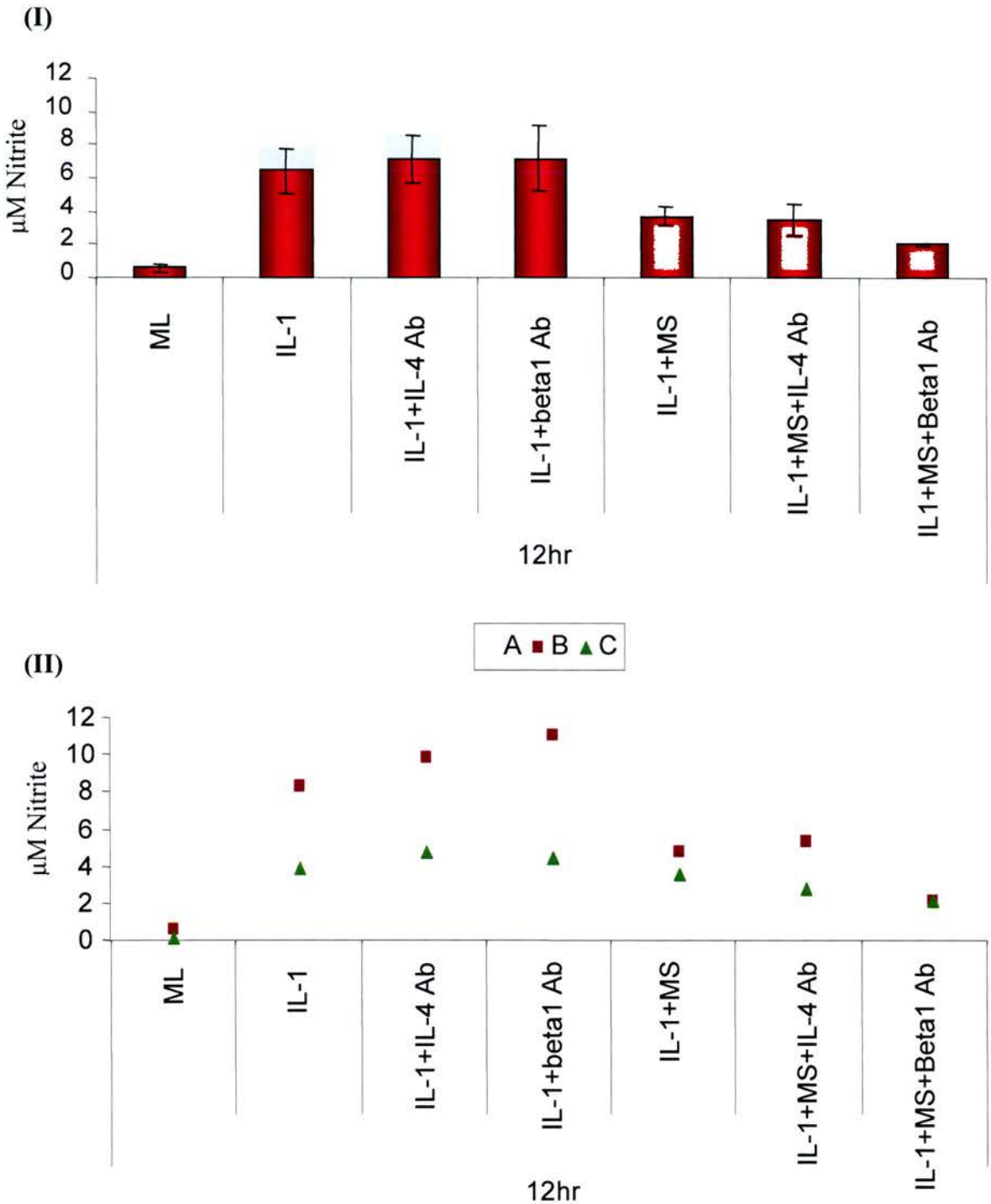


Figure 4.29 – mRNA adjusted nitrite data from Griess assay following incubation with IL-1β, β1 integrin blocking antibody and monoclonal IL-4 neutralising antibody. (I) = Pooled data±SEM. (II) = Individual experimental values.
ML = Unstimulated control. IL-1 = IL-1β stimulation (500pg/ml). Beta1 Ab = β1 integrin blocking antibody (1µg/ml). IL-4 Ab = IL-4 neutralising antibody (1µg/ml). MS = Mechanical stimulation. A=6/4/04 B=9/4/04 C=18/4/04. (n=3)

4.2.8 – Effects of the iNOS inhibitor AR-C102222 on CYT induced iNOS

The effects of a novel iNOS inhibitor AR-C102222 (10^{-6}, 10^{-5} and 10^{-4}M) on CYT induced iNOS mRNA and protein levels and enzyme activity in primary OA HAC was investigated.

Compared to the unstimulated control, the iNOS mRNA levels were not altered by the inhibitor at 10^{-6}, 10^{-5} or 10^{-4}M at 4 or 12 hours (**Figure 4.30**). CYT increased iNOS mRNA levels above unstimulated controls at both 4 and 12 hours ($p=0.0006$). The inhibitor at 10^{-6}, 10^{-5} and 10^{-4}M did not influence CYT stimulated iNOS mRNA levels at 12 hours. However, at 4 hours the 10^{-4}M concentration of inhibitor had a 66% lower level of iNOS mRNA than following CYT alone, or CYT in combination with 10^{-5} and 10^{-6}M inhibitor ($p=0.005$).
The inhibitor alone at 10^{-6}, 10^{-5} and 10^{-4}M did not alter iNOS protein production from unstimulated control levels at 4 or 12 hours (**Figure 4.31**). CYT stimulation increased iNOS protein levels above unstimulated control levels at both 4 and 12 hours. The inhibitor at 10^{-6}, 10^{-5} and 10^{-4}M had no significant effect on iNOS protein levels following CYT stimulation at 4 or 12 hours.
Nitrite accumulation in the medium was below 0.3μM at 4 hours following all the stimuli, and at 12 hours in the unstimulated control. This is at the limits of detection for the assay. Following 12 hour CYT stimulation nitrite levels were significantly increased above unstimulated controls to 3.7 and 6.2μM in mRNA- and protein-adjusted experiments respectively ($p=0.03$) (**Figures 4.32 and 4.33**). The inhibitor alone at 10^{-6}, 10^{-5} and 10^{-4}M did not alter nitrite levels from unstimulated control values. The inhibitor in combination with CYT resulted in lower levels of nitrite than those seen following CYT alone. There was a 96% and a 98% decrease at 10^{-4}M and 10^{-5}M and a 4.8 and 5.2 fold decrease at 10^{-6}M in mRNA- and protein-adjusted experiments respectively ($p\leq0.03$ for 10^{-4} and10^{-5}M and $p\leq0.057$ for 10^{-6}M). No significant difference was seen between the unstimulated control and the CYT nitrite levels when 10^{-4} and 10^{-5}M inhibitor is present ($p\geq0.072$). 10^{-6}M inhibitor in combination with CYT, however, resulted in a 2.3 fold and 5.1 fold elevation in nitrite levels from unstimulated controls in mRNA- and protein-adjusted experiments respectively ($p=0.045$ and $p=0.057$). These were 79% and 81% lower than those following CYT stimulation alone.

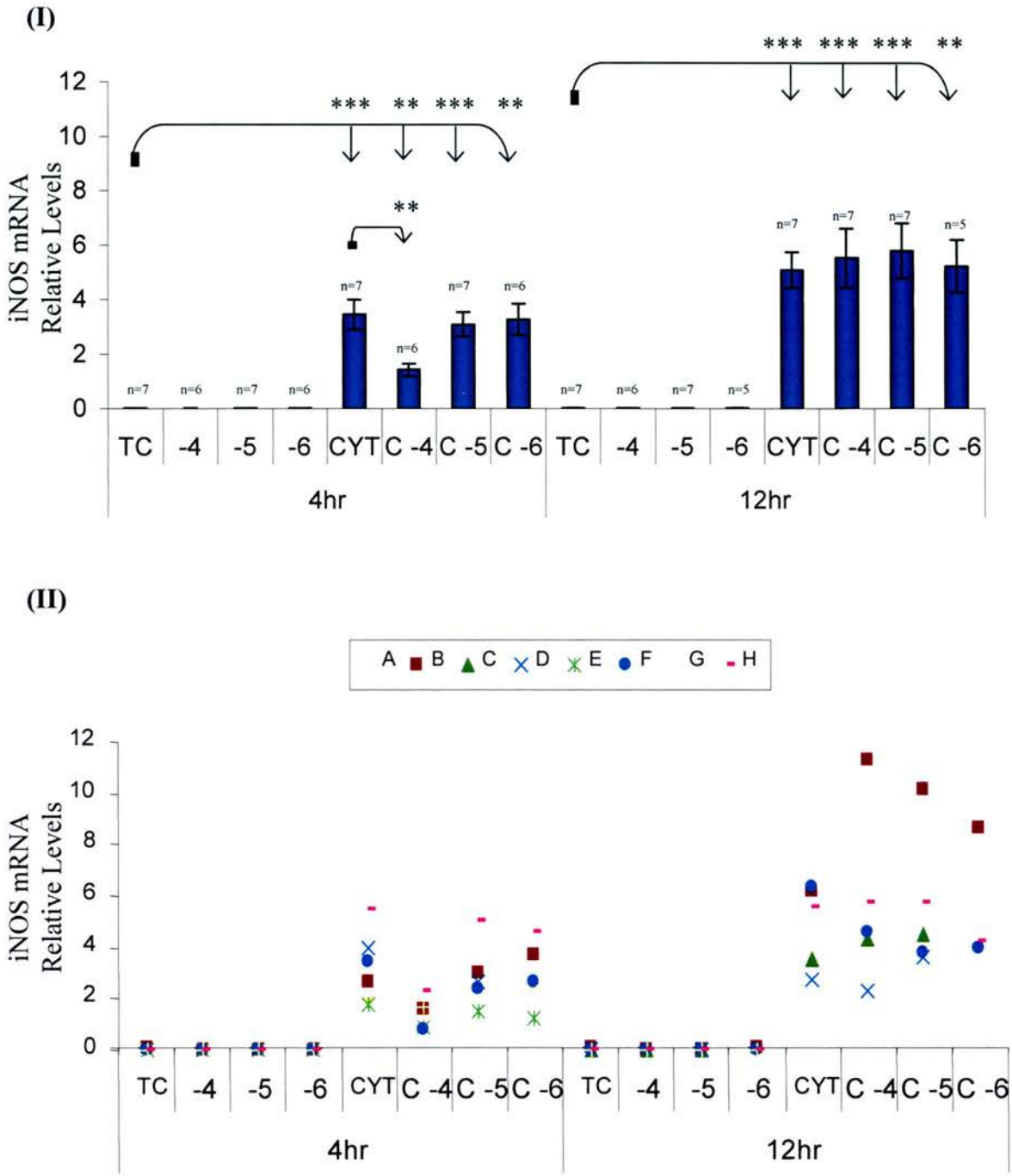


Figure 4.30 - iNOS mRNA levels following incubation with CYT and the iNOS inhibitor AR-C102222.
(I) = Pooled data±SEM. (II) = Individual experimental values.
TC = Unstimulated control. CYT and C = cytokine cocktail stimulation. –4 = 10^{-4}M Inhibitor. –5 = 10^{-5}M Inhibitor. –6 = 10^{-6}M Inhibitor.
A=DS004/8c B=DS008/9c C=6/12/02 D=6/5/03 E=17/6/03 F=18/4/04 G=11/5/04 H=21/5/04. **p<0.01 ***p<0.001.

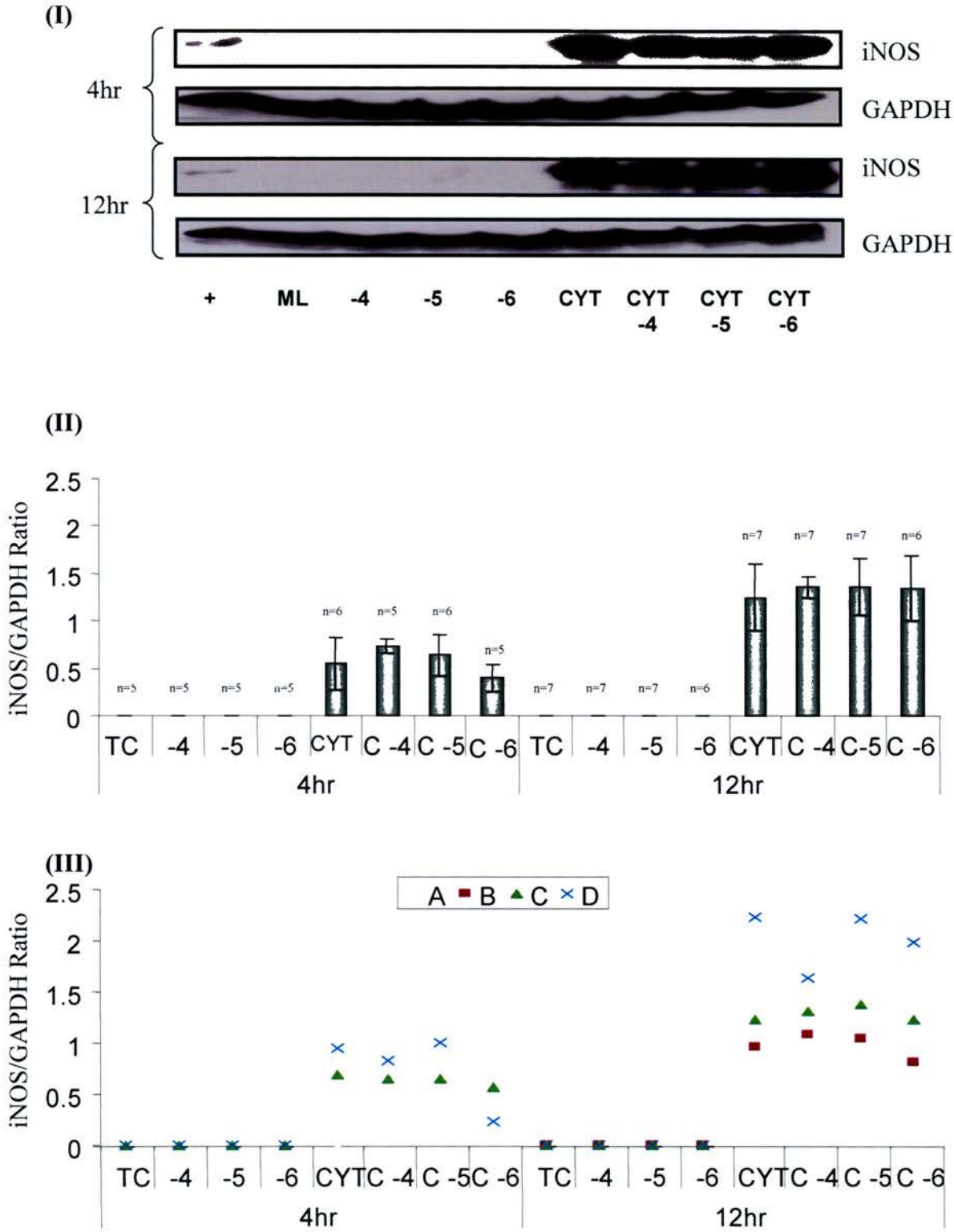
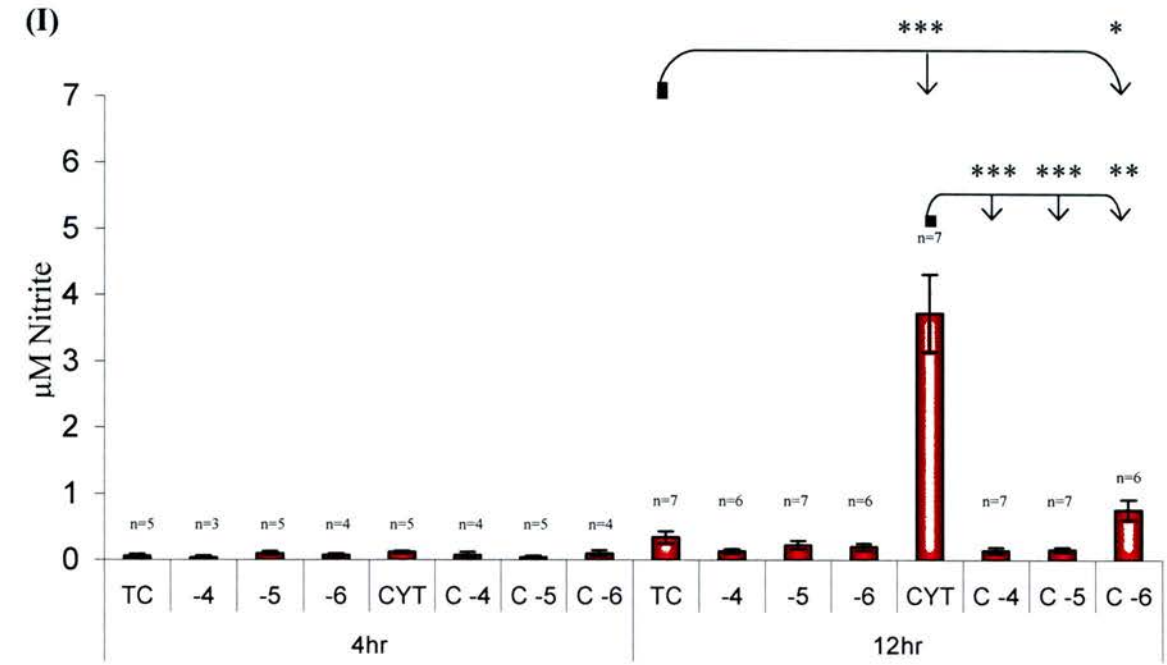


Figure 4.31 - iNOS protein level densitometry from western blots following incubation with CYT and iNOS inhibitor AR-C102222. (I) = Western blot (004). (II) = Pooled densitometry data±SEM. (III) = Individual densitometry experimental values. TC = Unstimulated control. CYT and C = cytokine cocktail stimulation. –4 = 10^{-4}M Inhibitor. –5 = 10^{-5}M Inhibitor. –6 = 10^{-6}M Inhibitor. A=6/5/03 B=6/12/02 C=DS004/8c D=DS008/9c.
Unable to compare TC and CYT as TC=0 so can not perform statistical analysis.



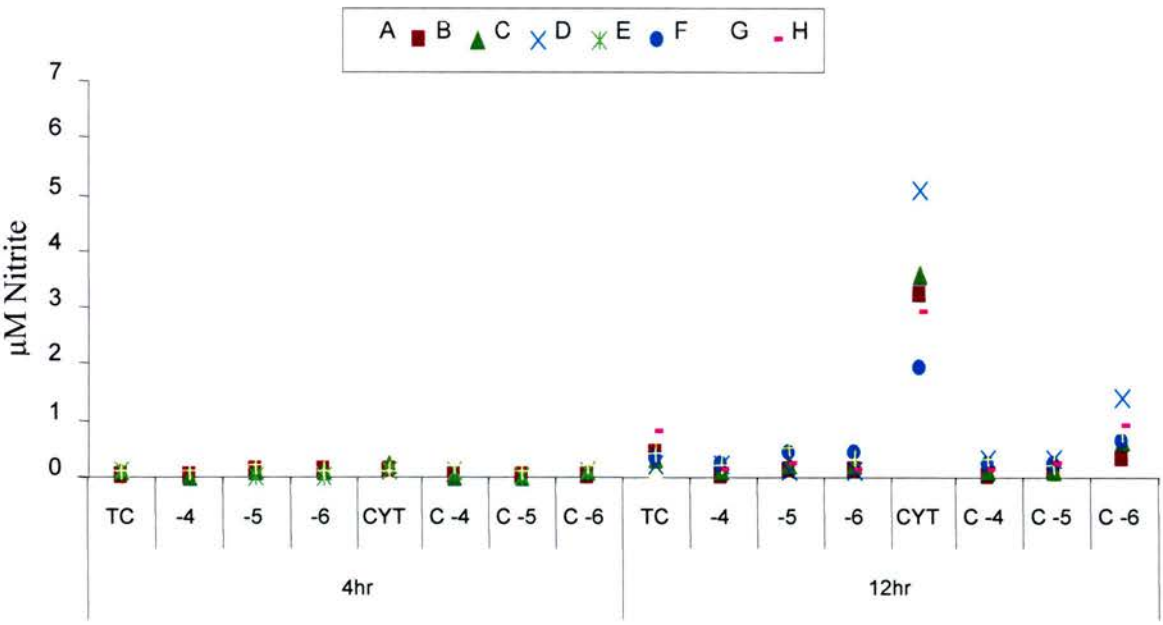


Figure 4.32 – mRNA adjusted nitrite levels from Griess assays following incubation with CYT and the iNOS inhibitor AR-C102222. (I) = Pooled mRNA adjusted data±SEM. (III) = Individual mRNA adjusted experimental values. TC = Unstimulated control. CYT and C = cytokine cocktail stimulation. –4 = 10^{-4}M Inhibitor. –5 = 10^{-5}M Inhibitor. –6 = 10^{-6}M Inhibitor. A=6/5/03 B=DS004/8c C=DS008/9c D=6/12/02 E=17/6/03 F=18/4/04 G=21/5/04 H=11/5/04. *p<0.05 **p<0.01 ***p<0.001.

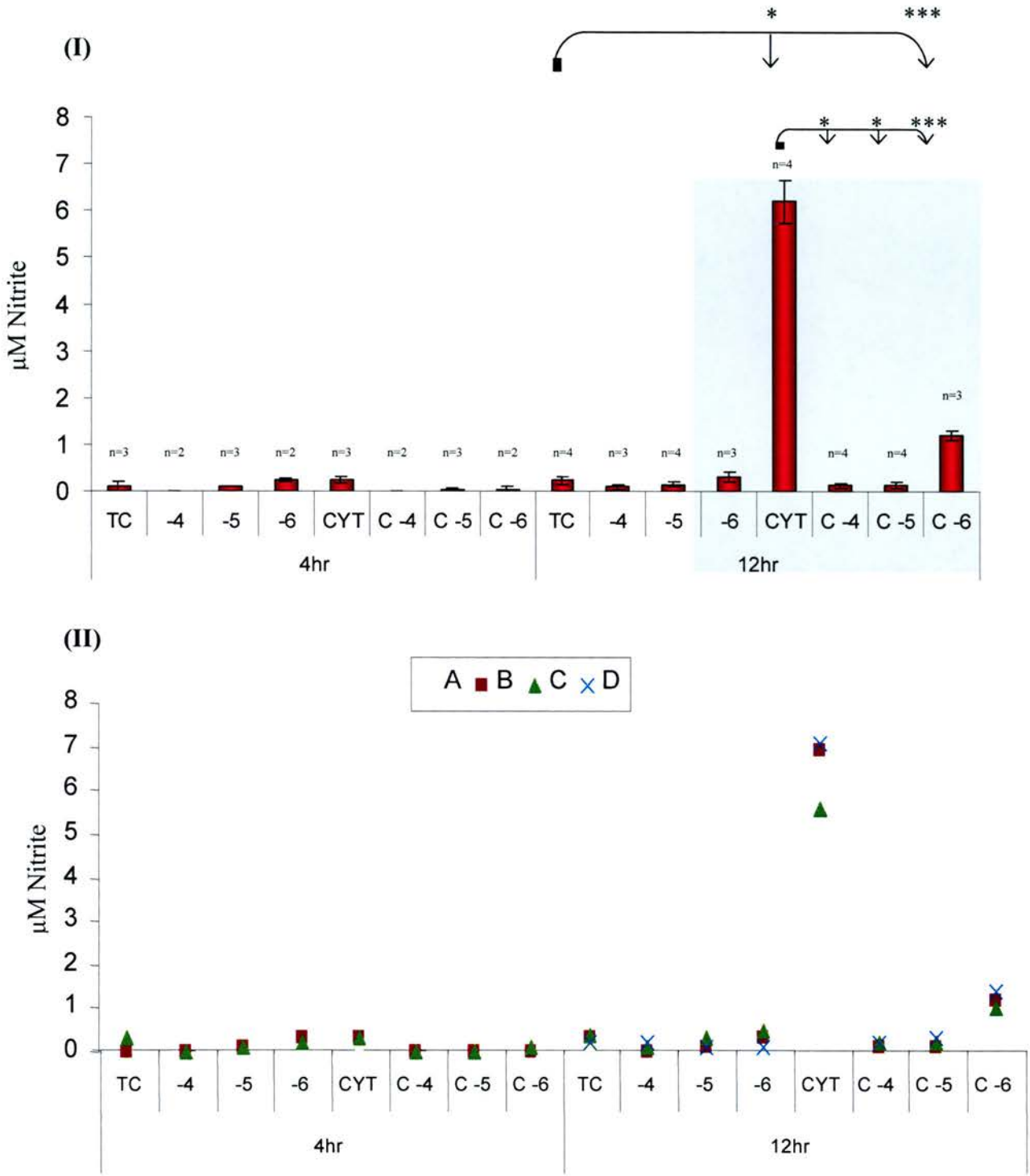


Figure 4.33 – Protein adjusted nitrite levels from Griess assays following incubation with CYT and the iNOS inhibitor AR-C102222. (I) = Pooled protein adjusted data±SEM. (III) = Individual protein adjusted experimental values. TC = Unstimulated control. CYT and C = cytokine cocktail stimulation. –4 = 10^{-4}M Inhibitor. –5 = 10^{-5}M Inhibitor. –6 = 10^{-6}M Inhibitor. A=6/5/03 B=DS004/8c C=DS008/9c D=6/12/02. *p<0.05 ***p<0.001.

4.3 – Discussion of results from mechanical and cytokine stimulation of primary OA HAC

Taqman real-time PCR analysis of primary OA HAC revealed that mRNA for e, n and iNOS was present at 12 hours in unstimulated cells. The increased cycle number required for detection of e and nNOS compared to iNOS suggests that there are lower levels of these mRNAs present in unstimulated cells. However, these levels can not be compared directly as the primer/probe efficiency will be different for each. Following incubation with CYT, iNOS mRNA levels were elevated several fold from unstimulated controls at 4 and 12 hours, whereas e and nNOS mRNA levels were decreased at 12 hours following CYT. The application of MS reduced the level of iNOS mRNA detected, but this was not statistically significant. However, the simultaneous application of CYT and MS significantly decreased the iNOS mRNA levels compared to those seen following CYT alone at both 4 and 12 hours, suggesting that MS influences CYT induced iNOS mRNA levels.

Protein analysis revealed that in unstimulated OA HAC, and following MS, e, n and iNOS were not detected at 4 or 12 hours. Following CYT, however, iNOS protein was detected at 4 hours and at an increased level at 12 hours. The simultaneous application of CYT and MS statistically decreased the level of iNOS protein detected compared to that seen following CYT alone at both 4 and 12 hours.

The inability to detect nitrite in the medium using the Griess assay at 4 hours, despite the presence of iNOS protein suggests that either the assay is not sensitive enough to detect small amounts of nitrite produced, or the protein detected at this time-point is not in an active state. The absence of nitrite formation could be due to the lack of the substrate, arginine, the level of which can be influenced by the activity of the enzyme arginase (Bogdan 2001). The lack of cofactors, such as tetrahydrobiopterin (BH_4), which are required for enzyme activity may also influence the level of nitrite produced (Tang et al 1995). Dimerisation is required for iNOS enzyme activity (Eissa et al 1998) and it may be that at 4 hours the enzyme has not yet taken a dimeric conformation so lacks enzyme activity. The protein detected in these experiments is denatured before being run on gels. It is therefore not possible to determine the conformation of the enzyme within the cell. It may also be that the small amount of NO produced at the 4 hour timepoint is being soaked up inside the cells by molecules such as glutathione (Kronke et al 2000), so preventing its release into the medium for

detection by the Griess assay. At 12 hours nitrite levels were elevated following CYT, and a statistically significant decrease in this level was detected following the simultaneous application of CYT and MS. These results suggest interaction between cytokine signalling and MS.

In order to elucidate the mechanism by which MS interferes with CYT induced iNOS levels two molecules involved in pathways induced by MS in HAC were blocked. The first of these, IL-4, is involved in the membrane hyperpolarisation associated with MS in normal HAC (Millward-Sadler et al 2000a; Millward-Sadler et al 2003). Initially IL-4 was blocked with a polyclonal IL-4 neutralising antibody. The lower concentration of this antibody (1μg/ml) did not influence iNOS protein levels following CYT plus MS. The higher concentration (10μg/ml) of the polyclonal antibody reduced the levels of iNOS protein and nitrite detected following CYT plus MS. However this concentration of antibody also decreased CYT stimulated iNOS protein and nitrite levels. This suggests that the antibody at the higher concentration is having an effect on cytokine signalling other than through blocking the action of IL-4. This may be due to the polyclonal nature of the antibody, allowing binding to different epitopes, some of which may be similar to other molecules on the surface of OA HAC, such as cytokine receptors, and thus causing interference with signalling. In order to try and eliminate this possibility a monoclonal IL-4 neutralising antibody was used. Incubation with this monoclonal antibody did not significantly alter the level of iNOS mRNA, protein or nitrite at 4 or 12 hours following either CYT alone or CYT plus MS.

α5β1 integrin has been implicated in the hyperpolarisation response that occurs in normal chondrocytes and depolarisation seen in OA chondrocytes following 0.33Hz MS (Millward-Sadler et al 2000a; Wright et al 1997). A β1 integrin function blocking antibody did not influence CYT or CYT plus MS stimulated iNOS mRNA, protein or nitrite levels at 4 or 12 hours. MS therefore appears to inhibit CYT mediated iNOS production by a β1 integrin and an IL-4 independent mechanism.

Although stimulation of primary OA HAC with a proinflammatory cytokine mix may mimic *in vivo* conditions to some extent, it also makes it difficult to elucidate the mechanism of action of MS, as each of the cytokines may induce different

intracellular signalling pathways that could be influenced by MS. The use of a single cytokine should therefore allow a better evaluation of the effect of MS. The stimulation of iNOS production due to IL-1β alone in primary OA HAC was evaluated and found to induce iNOS mRNA, protein and nitrite in a dose dependent fashion. MS decreased the iNOS mRNA and nitrite levels induced by IL-1β, although the protein response did not appear to be altered by MS when looking at the densitometry data. Densitometry can be misleading as the level of protein present and the intensity of the band detected do not follow a linear relationship. Analysis of the films produced from the western blots themselves suggests that there is a decrease in iNOS protein following MS. Incubation with both a monoclonal IL-4 neutralising antibody and a β1 integrin function blocking antibody had no effect on iNOS mRNA, protein or nitrite levels at 4 and 12 hours following IL-1β alone or IL-1β plus MS. This suggests that MS influences IL-1β in a β1 integrin and IL-4 independent fashion.

This study suggests that mechanical strain does not decrease CYT stimulated iNOS levels through a β1 integrin or IL-4 mediated pathway. It is necessary therefore to consider the other possible mechanisms for the action of strain in this system. It is possible that the strain is being sensed through a mechanoreceptor other than the β1 integrin. Other integrin molecules have been identified on the surface of chondrocytes including α1β1, α3β1, α5β1, α10β1, αvβ3 and αvβ5 (Knudson and Loeser 2002; Loeser et al 1995) and could be responsible for mechanotransduction. There are also other non-integrin molecules on the chondrocyte surface that could sense the mechanical strain. CD44 is one such molecule. It is a receptor for hyaluronan, which binds and aggregates aggrecan molecules. Serine phosphorylation of the intracellular domain of CD44 has been demonstrated, in addition to association with actin binding proteins such as ankyrin, suggesting that CD44 could be involved in signal transduction (Knudson and Loeser 2002). Indeed, the addition of hyaluronan to cartilage explants inhibits IL-1β mediated release of MMPs, a response which is reversed through the blocking of CD44 with specific antibodies (Julovi et al 2004). The fact that mechanical stimulation in our study not only decreased IL-1β mediated iNOS, but also the cytokine mix induced iNOS in primary OA HAC suggests a more general mechanism of action as several cytokine signalling pathways are blocked rather than just one. However, the only parameter measured is iNOS, so it may be that an iNOS specific mechanism is responsible for the action of MS on cytokine-

induced iNOS, while the other effects of the cytokines are unaffected. MS may influence cytokine signalling through the activation of stress activated ion channels that can modify the activity of signalling molecules inside the cell. Activation of potassium channels can activate proteases in the phagocytic vacuole of neutrophil leukocytes through increased calcium concentration and membrane depolarisation (Ahluwalia et al 2004), both of which occur following MS in our system (Wright et al 1997). By immunostaining Shakibaei and Mobasheri (2003) showed that in mouse limb bud chondrocytes β1 integrins colocalise with a Na K-ATPase, epithelial sodium channels and a voltage activated calcium channel. This would allow control of calcium and sodium influx and so influence the activation of intracellular molecules. Opening of these ion channels may also alter the pH within the cell. A chondrocyte cell line study showed that alkalinisation following loading resulted in increased inositol triphosphate (IP_3) levels and the release of calcium (Ca^{2+}) from intracellular stores, so potentially influencing cell signalling (Browning and Wilkins 2002). Studies by our group have shown the importance of phospholipase C (PLC) following mechanical stimulation where IP_3 mediated Ca^{2+} release is required for membrane hyperpolarisation in normal chondrocytes and membrane depolarisation in OA chondrocytes (Millward-Sadler et al 2000a; Wright et al 1997).
The function of integrins can be modulated by the presence of integrin associated proteins such as CD47. CD47 has been shown to associate with αIIbβ3 and α4β1 and modulates the function of beta β1, beta β2 and beta β3 integrins through G protein activation (Brown and Frazier 2001; Fujimoto et al 2003; Li et al 2002).

The downregulation of cytokine receptors is another mechanism through which mechanical stimulation could decrease iNOS production. However, cyclic tensile strain applied before the application of IL-1β has only a small effect on iNOS mRNA and nitrite levels, suggesting that IL-1β receptor downregulation is unlikely to be the mechanism through which strain acts to decrease iNOS levels (Xu et al 2000). Stimulation with IL-1β one hour prior to the application of cyclic tensile strain halves the response and there is no decrease in iNOS mRNA or nitrite due to strain applied 2 hours after IL-1β (Xu et al 2000). This suggests that cytokine signalling has already initiated iNOS gene transcription and the application of mechanical strain has no effect on this. It may also be that the prior application of cytokine signalling interrupts molecules involved in mechanical signalling, preventing the effects of

mechanical strain. The production of IL-4 may stimulate the release of IL-1ra, although it has been reported that IL-4 does not stimulate IL-1ra production in human articular chondrocytes, and that IL-1β induced IL-1ra takes 24 hours to be expressed (Palmer et al 2002). This suggests that IL-1ra production is unlikely to influence the production of iNOS during our 12 hour experimental regime.

Another important observation from these results is that within individual experiments samples can respond differently to each of the stimuli. This may be influenced by many factors. Differences in proteoglycan synthesis have been shown to be dependent upon the region of the articular cartilage from which the chondrocytes were derived (Wiseman et al 2003). The pattern of integrin expression in chondrocytes is also dependent on the degree of cartilage damage, suggesting that cells extracted from different grades of OA cartilage will sense strains differently (Iannone et al 2001). Cartilage from different joints have different properties that influence the chondrocyte response to mechanical strain (Xu et al 2004). Increasing age of the donor cartilage influences the response of the chondrocytes to IL-1β stimulation, with less generation of iNOS with increasing age (Min et al 2001). Oestrogen may antagonise the production of iNOS in chondrocytes. Oestrogen production is decreased in menopausal females suggesting that gender related differences will influence the response of chondrocytes to cytokine stimulation (Cuzzocrea et al 2003). The treatment a patient with OA may have been receiving is also important. NSAIDs influence the production of inflammatory mediators from chondrocytes, and so can influence their response to proinflammatory cytokine stimulation (Sanchez et al 2002).

The differentiation state of these cells during experimentation should also be considered. Primary chondrocytes are known to dedifferentiate in monolayer culture, resulting in expression of different ECM molecules and integrins which can influence the way in which strains are sensed (Mahmood et al 2004). This altered pattern of ECM synthesis can be partially reversed by 3-d culture in alginate (Yoon et al 2002). However, dedifferentiation was not an issue in my experiments as culture in monolayer was limited to less than 10 days. At this time point HAC continue to express chondrocyte phenotypic markers such as collagen type II and aggrecan and continue to maintain their integrin expression profile (Edinburgh University OA

research group unpublished observations). The relative lack of ECM produced by cells in monolayer cells is also important as it will allow better diffusion of the cytokines added. It may however also allow better diffusion of signalling molecules secreted by the cells, so causing a more general effect than might be seen in cartilage itself where the ECM restricts the diffusion of signalling molecules.

Potential interactions between mechanotransduction and cytokine signalling pathways (Figure 4.3.2)

In order to understand how MS may be interfering with CYT signalling it is first necessary to understand what is known about the signalling mechanism of each of these stimuli individually.

The mechanism by which chondrocytes sense mechanical signals has been discussed in detail in Chapter 1 (**Figures 1.7 and 1.8**). Briefly, integrins on the surface of chondrocytes bind components of the ECM and transduce mechanical signals to an intracellular signalling complex, the focal adhesion complex (FAC). This contains the actin cytoskeleton and tyrosine kinases such as focal adhesion kinase (FAK), paxillin and β-catenin that are activated by mechanical stimulation (Lee HS et al 2000). The activated kinases initiate intracellular signalling cascades involving PLC and protein kinase C (PKC) that result in altered gene transcription (Millward-Sadler et al 2000b). The activation of calcium dependent potassium ion channels by mechanical signals is a response involving the autocrine/paracrine production of soluble mediators, such as substance P and IL-4, leading to the measured hyper and depolarisation responses seen in normal and OA HAC respectively following MS (Millward Sadler et al 2000a; Millward-Sadler et al 2003). Much of the work in cartilage has focussed on the outcomes of mechanical stimulation, such as altered proteoglycan synthesis (discussed in Chapter 5) and relatively little is known about the intracellular signalling cascades leading to these responses.

Cytokine signalling, also discussed in Chapter 1 (**Figure 1.3**), occurs through the binding of the soluble cytokine molecules to their receptors on the cell surface. Intracellular signalling events occur upon cytokine binding, with each cytokine using different signalling pathways. IL-1β functions through the activation of the upstream regulator of mitogen activated protein kinase (MAPK) activity TAK1, which can

control the activation state of the transcription factor NFκB (Slack et al 2000). TNFα signals through the activation of the upstream regulator of MAPK activity MEK1/2 and NFκB activation (Seguin and Bernier 2003). The phosphorylation of the Janus activated kinases (JAKs) attached to the IFNγ receptor (Watling et al 1993) activates p91 which moves to the nucleus where it binds the gamma activated sequence (GAS) on gene promoter regions (Shuai et al 1992). IL-6 activation causes Janus activated kinase (JAK) and Tyk2 phosphorylation, activating the signal transducer and activator of transcription (STAT) 3 that binds the promoters of certain genes (Stahl et al 1994). Each of the cytokines can therefore influence gene transcription patterns. There is, however, crosstalk between these signalling pathways, as highlighted by gene deletion studies which reveal that few individual cytokines are absolutely essential for life or even for individual cellular functions, suggesting that one cytokine can compensate for the loss of another (Ozaki and Leonard 2002).

Growth factors and cytokines affect integrin expression and matrix affinity, and so can influence integrin signalling (Giancotti and Ruoslahti 1999; Jobanputra et al 1996). This may occur through direct alteration of the integrins expressed; expression of the α5 integrin subunit is increased following cyclic compressive stress in bovine articular explants (Lucchinetti et al 2004), while alpha2 and alpha5 integrin subunits are increased in human articular chondrocytes cultured in monolayer on flexible membranes and subjected to cyclic strain for 24 hours (Lahiji et al 2004). Alternatively, integrin signalling can be influenced through the production of an altered ECM that allows the cells to sense mechanical signals differently (Loeser 1997). The reverse is also true. Cytokine signalling pathways can be influenced by integrin activation, as illustrated by the requirement for α5β1 integrin binding to fibronectin to allow a rabbit chondrocyte proliferative response following fibroblast growth factor (FGF) stimulation (Enomoto-Iwamoto et al 1997). Interaction between cytokine and mechanical signalling molecules would appear unlikely if the receptors were signalling through distinct points at the cell membrane surface. However, there is evidence suggesting a close association between these cell surface receptors, and some of the intracellular signalling molecules may be activated by either stimulus. Several cytokine and growth factor receptors that have an association with FAC have been identified, including the IL-1RI (Lo et al 1998) and the insulin receptor (Vuori and Ruoslahti). It has been suggested that interaction with the integrin complex

causes partial activation of growth factor receptors, which are then fully activated following ligand binding (Giancotti and Ruoslahti 1999).

Load alters the physical environment of chondrocytes in a complex fashion as discussed in Chapter 1. The tissue and cells deform, leading to a rise in the hydrostatic pressure of the matrix, and fluid is expressed in order to restore osmotic equilibrium. Increases in extracellular osmolarilty, ion concentration, and the fall in pH that follows, can influence chondrocyte metabolism as can be seen as proteoglycan synthesis decreases following static load (discussed in Chapter 5) (Urban et al 1993). Other factors such as the alteration of oxygen levels during exercise, which is known to affect matrix synthesis and cell growth may also be important (Marcus 1973). Mechanotransduction through cell surface receptors may influence chondrocyte function. However, cell deformation and associated distortion of intracellular organelles may also influence the cells response to a mechanical stimulus (Knight et al 2002). Indeed, pressures in the range of 5-50MPa may alter cell morphology, reduce exocytosis, dissociate cytoskeletal elements, reduce protein synthesis and inhibit membrane transport (Hall et al 1991).

It has been suggested that the application of mechanical stimulation to cartilage results in release of ECM attached growth factors that influence the activity of the chondrocytes (Vincent et al 2004). However, in this system the cells are in monolayer culture and produce only a limited ECM, and so are unlikely to be affected in this way. It has also been suggested that mechanical stimulation affects chondrocytes by speeding the rate of diffusion of soluble factors from the surrounding medium in explant studies (Bonassar et al 1997; Jin et al 2003) and induces solute movement through cartilage which can alter cellular metabolism (O'Hara et al 1990). This is also unlikely to be an important factor in these experiments where the cells are in monolayer and express a limited ECM.

It is also possible that mechanical stimulation could activate a transcription factor by binding to a mechanoresponsive element. An example of this is seen where Tenascin-C is upregulated by mechanical stimulation. A mechanoresponsive element in the Tenascin-C promoter was identified and found to be present in the collagen type XII promoter also, although the transcription factor that binds to it has yet to be identified

(Chiquet et al 2003). It is also possible that strain acts by inducing the synthesis of a nuclear factor which then activates ECM genes, such as the EGR-1 gene (Schwachtgen et al 1998). Mechanical strain may also act through the induction of the synthesis or secretion of soluble factors that regulate ECM gene expression via an autocrine/paracrine mechanism.
It is therefore worth considering, given that mechano-responsive elements exist, that the outcome following mechanical and cytokine stimulation is the result of a cumulative effect of both the positive and negative factors on the promoters of genes that define their rate of synthesis. Indeed, considering the complexity of regulation of the human iNOS promoter (discussed in chapter 1), and the interactions between transcription factors it is possible that this may be the case.

An example of a mechano-responsive transcription factor is NFκB, which has been activated following mechanical stimulation in a manner that is dependent upon the magnitude of the signal applied. Agarwal et al (2003) initially showed this using low and high magnitudes of strain on osteoblast like periodontal ligament cells, subsequently showing the same effect in lapine temperomadibular junction chondrocytes in monolayer (Agarwal et al 2004). These studies showed the low strain inhibits IL-1β induced NFκB nuclear translocation, stabilising the IκB/NFκB complex, while high strain mimics IL-1β and induces NFκB nuclear translocation. As discussed in Chapter 1, NFκB is a major transcription factor involved in the synthesis of iNOS following IL-1β stimulation of chondrocytes, so its inhibition is a mechanism by which strain could be causing its effects within our experiments following single cytokine stimulation. The inhibition of iNOS production when the cytokine cocktail is added could also be explained by this phenomenon, however, it is also likely that control of such a response would need to be more general, such as through the activation of ion channels, rather than through the integrins alone. This is supported by our finding that the β1 integrin function blocking antibody does not prevent the effect of mechanical strain, suggesting an alternative mechanism of action.

IL-1RI colocalisation with vinculin at sites of fibronectin attachment suggest it can be recruited to the FAC. RhoA is involved in FAC formation and is associated with ligand bound IL-1 receptor and activated by IL-1β, confirming the interaction of IL-RI and FAC (MacGillivray et al 2000). Increased activation of signal transduction

pathways and enhanced release of inflammatory mediators is seen when the IL-1RI is localised to FAC in human gingival fibroblasts (Huang et al 2001). Association of IL-1RI with the G proteins Ras and Rac occurs in the FAC and may represent a method of enhanced signal transduction (O'Neill 2002).

MAPK

The MAPKs (**Figure 4.3.1**) are of interest as they are activated both by mechanical and cytokine signalling (Fanning et al 2003; Mendes et al 2002). These molecules require threonine and tyrosine phosphorylation in order to activate them, which ensures they remain inactive within the cell unless the appropriate stimulation is received.

MAPK activation by mechanical stimuli has been investigated in chondrocytes and in other cell types. Compression of cartilage explants was shown to phosphorylate extracellular signal regulated kinases (ERK) 1 and 2, p38 MAPK and stress activated protein kinase (SAPK)/ERK (SEK-1) of the c-Jun NH_2 terminal kinase (JNK) pathway in a time dependent manner (Fanning et al 2003). Fluid flow in bovine articular chondrocytes increases ERK1/2 activation at a shear stress of 1.6Pa (Hung et al 2000). Studies in other cell types show activation of ERK, p38 and JNK due to mechanical strain in different systems, an example of which is seen during 4% stretch of rat cardiac myocytes where increased ERK2, JNK1 and p38 MAPK activation occurs via an integrin dependent mechanism (MacKenna et al 1998).
Studies using 0.33Hz MS within our system have shown that JNK phosphorylation and activation, which is partially integrin-dependent, occurs following MS in chondrocyte cell lines, while p38 and ERK1/2 remain unaffected (OA research group, Edinburgh University, submitted for publication). The presence of p38, ERK and JNK has been identified in primary OA HAC, with increased p38 phosphorylation following MS that is $\alpha5\beta1$ integrin-independent (OA research group, Edinburgh University, unpublished observations). This supports an alternative mechanism of mechanical signalling in our experiments leading to the decreased CYT induce iNOS mRNA, protein and nitrite observed. The transient association of intracellular receptor activated kinase (IRAK) with the FAC in IL-1β stimulated human gingival fibroblasts is involved in IL-1β-induced ERK activation, a response that requires an intact cytoskeleton. However, in the absence of the FAC, IL-1β still activates p38 and JNK.

This suggests that the cytoskeleton may be the scaffold for ERK and other signalling molecules to be brought together to allow ERK phosphorylation at the FAC following exposure to IL-1β (MacGillivray et al 2000).

The activation of MAPKs has also been demonstrated following cytokine and growth factor stimulation. p38 MAPK phosphorylation and activation is required for iNOS mRNA, protein and NO production in bovine articular chondrocytes following IL-1β stimulation, whereas ERK1/2 is not required (Mendes et al 2002). The activation of p38 and NFκB are both required for IL-1β induced iNOS production, signalling through distinct pathways. The MAPK signalling cascade has, however, been implicated in NFκB signalling, through the upstream kinase involved in MAPK activation, MAPK and ERK kinase (MEKK) 1. MEKK1 is suggested to be part of the IκB/IKK/NFκB complex, causing phosphorylation and activation of IκB kinase (IKK) which phosphorylates the inhibitor of NFκB, IκB, targeting it for degradation. This exposes the nuclear localisation sequence on NFκB allowing it to travel to the nucleus and influence gene transcription (Hagemann and Blank 2001). The transcription factor AP-1 contains the subunit c-Jun that is a direct target for JNK, which is activated through TNF receptors via a MEKK1-SEK-JNK module using a similar mechanism to NFκB, with MEKK1 being recruited to the receptor complex (Hagemann and Blank 2001).

The role of MAPK in the production of iNOS could be explained by decreased mRNA stability due to strain (Sironen et al 2002). It has been suggested that ERK does not regulate iNOS mRNA expression but possibly influences post-transcriptional events such as mRNA stabilisation (Wang and Brecher 1999). It is possible that this may be due to decreased post translational processing, allowing AU rich regions to remain, or could involve the synthesis of regulatory proteins such as HuR (Rodriguez-Pascual et al 2000).

The phosphorylation of MEKK1 is essential for its catalytic activity and this requires direct upstream kinases. It has been suggested that protein kinase C (PKC) phosphorylates MEKK1, leading to JNK activation (Siow et al 1997). This is of particular interest as PKC is involved in the mechanotransduction response to 0.33Hz MS. PKCα, gamma, delta, iota and lambda have been identified in human articular

chondrocytes (Lee HS et al 2002), with the translocation of PKCα to the FAC through interaction with receptor for activated c-kinase (RACK-1) and association with the β1 integrin. Loeser et al (2003) showed that a fibronectin fragment and an α5β1 integrin activating antibody stimulate PKC activation, causing proline rich tyrosine kinase 2 (PYK2) activation and MAPK stimulation and resulting in the upregulation of MMP13 expression. Cheng et al (2001) showed that cyclic strain applied to endothelial cells resulted in PKCα activation followed by PKCε activation, causing sustained Raf/ERK1/2 activation and stimulation of Elk1 promoter activity. Mesangial cells subjected to cyclic strain showed rapid SAPK/JNK activation that was dependent on increased intracellular calcium and PKC, which activates Elk-1, stimulating c-fos production and leading to AP-1 activation and increased fibronectin mRNA levels (Ingram et al 2000a).

PKC activation is also seen as a result of growth factor and cytokine signalling in response to TNFα (Hagemann and Blank 2001), vitamin D (Maeda et al 2001) and histamine (Sohen et al 2001). TGF-β addition to rat chondrocytes causes increased proliferation and proteoglycan synthesis. This occurs via the stimulation of G proteins that activate phospholipase A2, causing PKA activation that activates PKC (Rosado et al 2002). PKC has also been suggested to have the ability to phosphorylate IκB, causing the release and nuclear translocation of NFκB (Steffan et al 1995). PKC induction due to strain can inhibit NO production due to IL-1β and IFNγ in rat cardiac myocytes (Yamamoto et al 1998).

It has also been suggested that PKC can phosphorylate ion channels, altering their activity and therefore influencing the intracellular environment of the cell. An example of this is where cyclic stretch upregulates the production of Na+, K+-ATPase in rat smooth muscle cells via a PKC dependent mechanism (Songu-Mize et al 1998).

The activation of G proteins has been demonstrated following both cytokine and mechanical stimulation of cells. G proteins are targeted to specific cell surface microdomains (Oh and Schnitzer 2001). Mechanical compression of cartilage results in changes in cell volume and calcium concentration oscillations. The use of pharmacological inhibitors of signalling molecules revealed that this response required gadolinium sensitive stretch activated ion channels, IP3 and G protein

activity (Erickson et al 2001). The activation of the small G protein Ras by fluid induced shear stress of human vascular endothelial cells was shown to be mediated through G proteins (Gudi et al 2003). Ras and RhoA have been implicated in the processing of mechanical signals to downstream signalling molecules such as ERK1/2 and protein kinase C (PKB) (Sugden 2003). G proteins have been implicated in the modulation of NO release from osteoblasts, where cyclic tensile strain activates the G protein which enhances JNK/SAPK activity and decreases NO through a mechanism that requires an intact cytoskeleton (Hara et al 2001).
P2Y(2) receptors are able to bind integrin subunits and through association with G proteins and CD47 can activate FAK, ERKs and initiate PLC dependent mobilisation of intracellular calcium (Erb et al 2001). Millward-Sadler et al (2004) showed the presence of P2Y2 purinoreceptors on normal and OA chondrocytes and that binding of ATP was sufficient to stimulate the membrane hyperpolarisation associated with MS in normal cells.

The question of how specificity is achieved within these pathways has to be addressed, especially when considering that the loss of one pathway can be compensated for by another. The N-terminal motifs of the MAPKs alter allowing different subcellular localisation. MEKK1 can bind α-actinin, locating it to focal adhesions which suggests it may be involved in mechanotransduction. Proteolytic processing of MEKK1 alters its function, possibly by allowing it to interact with different pools of signalling molecules, and this has been implicated in IL-1β (Kopp et al 1999) and TNFα (Fanger et al 1997) mediated NFκB and AP-1 activation. Binding of MEKKs to regulatory proteins can alter specificity, seen in TNFα signalling where Gck links MEKK1 to JNK activation, whilst Tax association allows preferential NFκB stimulation (Shi and Kehrl 1997; Yin et al 1998; Yuasa et al 1998). 14-3-3 proteins associate with MEKKs and have a scaffold function, bringing attached factors together as well as acting as chaperones, binding serine phosphorylated proteins and preventing their dephosphorylation (Fanger et al 1998). This allows the control of MEKK function through mediating interactions with other proteins and controlling subcellular localisation. Other scaffold proteins have also been identified that bind MAPK module members, bringing them together and so influencing the pathways activated. MEKK1 itself acts as a scaffold for Raf-MEK-ERK2, and since it is also associated with the cytoskeleton, it could keep the signal

module close to FAC so allowing efficient cellular responses to MS (Karandikar et al 2000). The different subcellular localisation of Raf alters its function from proliferative to pro-apoptotic (Wang et al 1996) so scaffold proteins can increase the specificity of signalling pathways activated, but they restrict the signal amplification that occurs through sequential kinase interactions.

Integrin mediated MAPK pathways converge with cytokine induced pathways (Sundberg and Rubin 1996), so will both have an effect on the regulation of cell function. The exact biological functions of these kinases will depend upon a number of factors including the nature, intensity and duration of upstream signals, the cellular localisation of the signalling proteins and crosstalk with other signalling pathways. The complicated interactions between the MAPK signalling pathways, activation of MEKK cleavage to alter its subcellular location, production of regulatory proteins, scaffold proteins or the activation of PKC activity are some of the ways in which mechanical strain could interfere with cytokine signalling through the MAPKs. It is also possible that mechanotransduction occurs at a faster rate than cytokine signalling, so uses up the signalling molecules in the FAC, leaving less available for cytokine signalling. This hypothesis is supported by the fact that cytokine stimulation for 1 hour prior to mechanical stimulation reduces the inhibitory response of strain on iNOS mRNA and nitrite production, and 2 hours before completely inhibits the response compared to when both stimuli are applied simultaneously (Xu et al 2000). It is necessary to identify and understand the function of the upstream regulators, downstream mediators and target genes of the MAPKs in chondrocytes in order to elucidate the mechanisms by which mechanical stimulation may influence the cytokine signalling cascade.

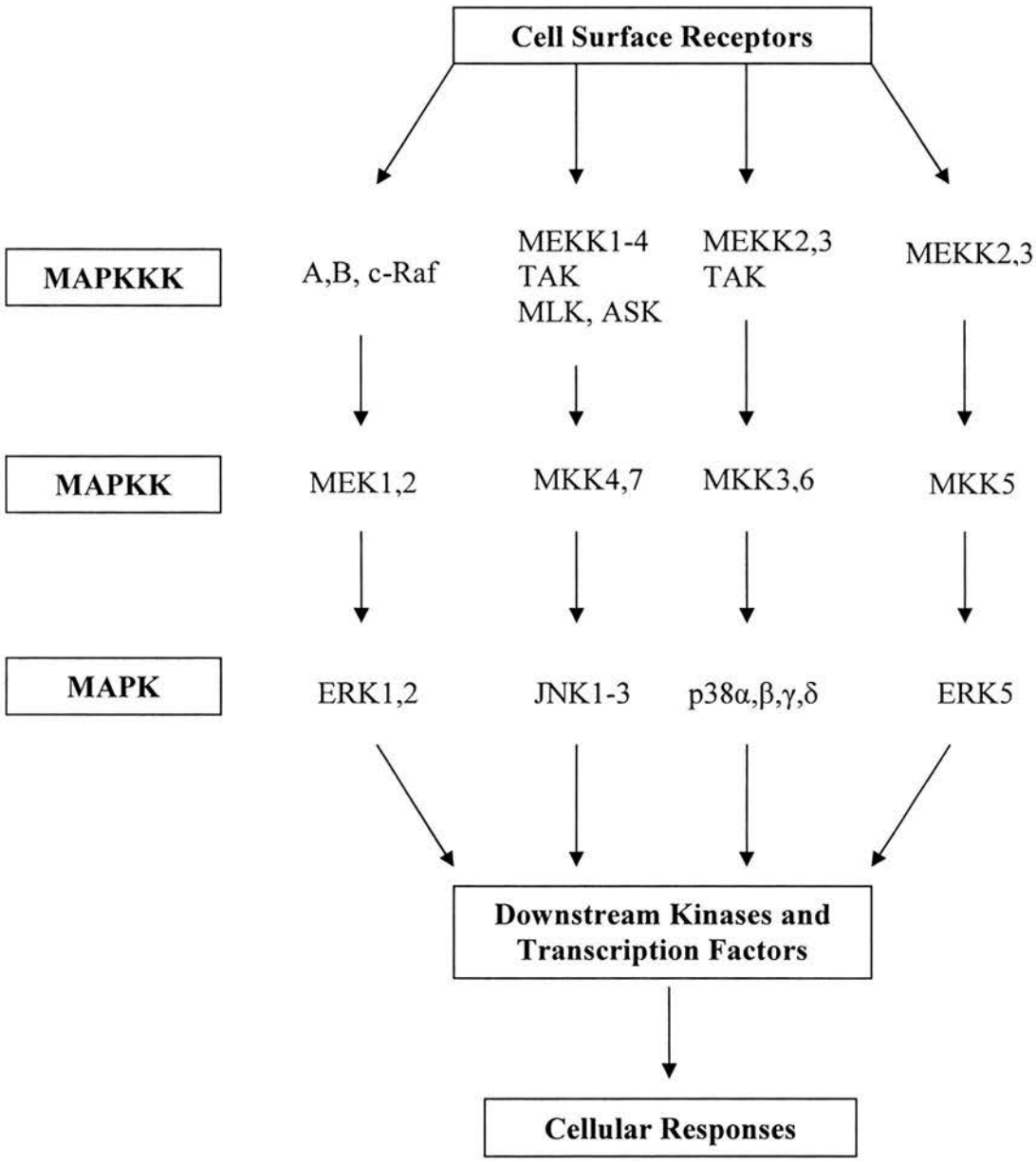


Figure 4.3.1 – MAPK pathways where receptor stimulation causes phosphorylation of MAPKKK, which phosphorylate MAPKK resulting in MAPK phosphorylation and activation. Phosphorylation of substrates and transcription factors causes specific responses through altered gene transcription. (Stanton et al 2003).

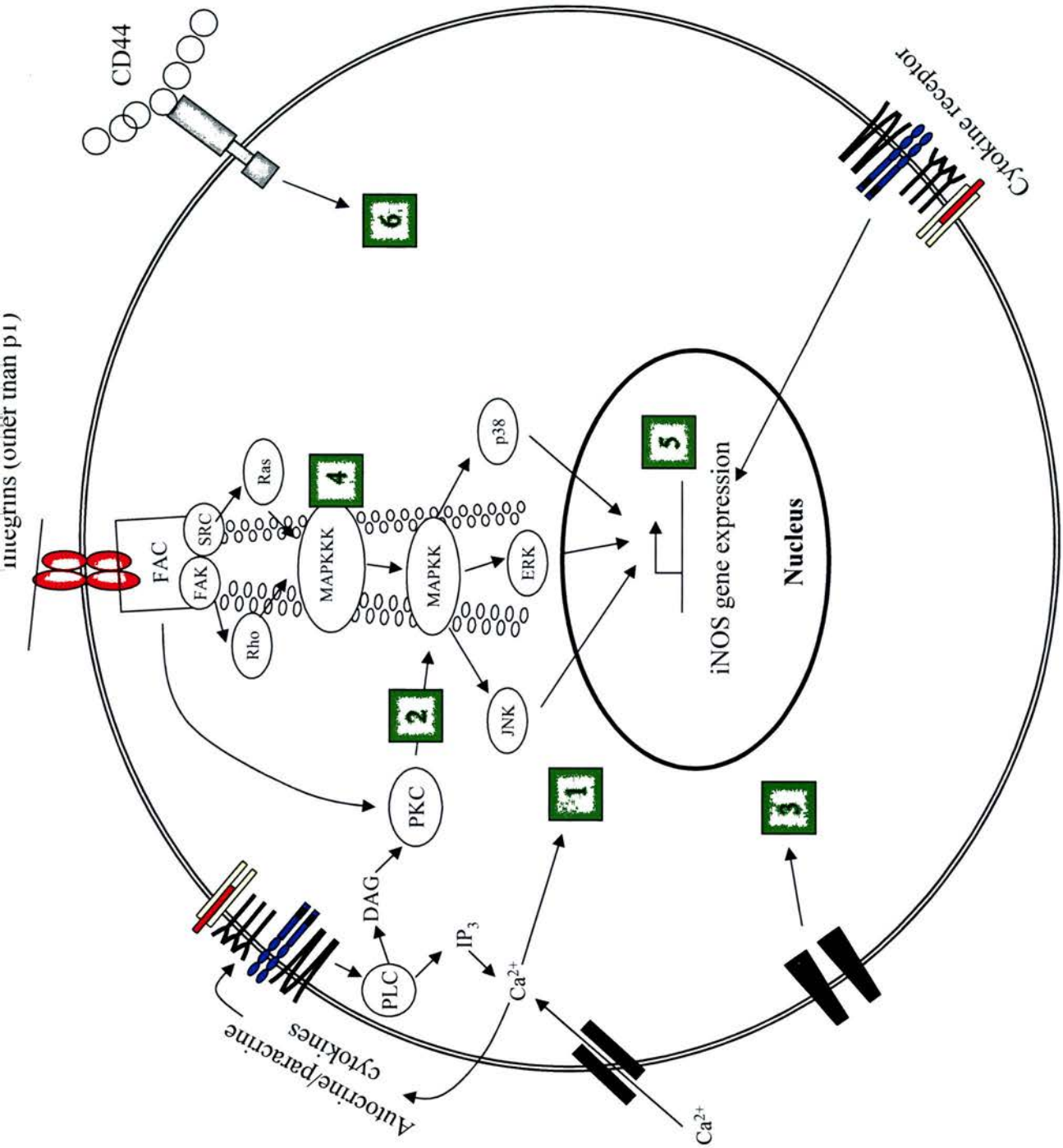


Figure 4.3.2 – Possible interactions between mechanical signalling and cytokine signalling in OA HAC. (1) Calcium increased through stretch activated ion channels and from intracellular stores can activate signalling molecules and may be responsible for stabilising the NFκB/IκB complex so inhibiting NFκB signalling. (2) PKC activated by both mechanical and cytokine signals and can influence MAPK signalling and transcription factor activation. (3) Activation of other stretch activated ion channels may influence intracellular signalling molecules. (4) The MAPKKK MEKK1 is involved in release of NFκB through activation of IKK. Its role in mechanotransduction may mean it is localised to a different cellular compartment so less activation of NFκB is seen following cytokine stimulation. (5) There may be positive and negative regulatory elements on the iNOS gene promoter, influencing the rate of its expression. (6) Other cell surface Molecules may sense the mechanical signals and influence cytokine signalling.

iNOS Inhibitor AR-C102222

The novel iNOS inhibitor AR-C102222 did not induce iNOS mRNA, protein or nitrite synthesis at 4 or 12 hours at any of the concentrations tested. The inhibitor did not significantly alter the level of iNOS mRNA and protein at 4 and 12 hours at the two lower concentrations (10^{-5}M and 10^{-6}M). However at the highest concentration used (10^{-4}M) iNOS mRNA levels decreased at 4 hours following CYT. The 18s mRNA levels detected remain at a similar level to those seen for the other treatment regimes, and the cell viability studies show no effect on cell death at this concentration (**Appendix 1 and 2**). This suggests that the inhibitor is not causing general downregulation of mRNA production, or decreasing the cell number and thus lowering the levels detected. It would appear more likely that the inhibitor is interfering with the actual iNOS mRNA synthesis or degradation at this concentration. AR-C102222 inhibits the increase in nitrite levels following CYT at both 10^{-4} and 10^{-5}M. However a small amount of nitrite is still produced at 10^{-6}M suggesting incomplete inhibition of enzyme activity at this concentration. These results suggest that 10^{-5}M may be the optimal concentration to use this inhibitor as it is high enough to completely inhibit enzyme activity without altering iNOS mRNA levels through an unknown mechanism.

Method of Inhibitor action

A high degree of structural conservation within the active site of the NOS enzymes makes it difficult to design isoform specific inhibitors (Crane et al 1998). Many of the inhibitors described interact with the active site where the substrate L-arginine binds. There are also several inhibitors of the cofactor tetrahydrobioterin (BH_4), as well as molecules that prevent NOS dimer formation. Inhibitors of electron transfer in the reductase domain can be effective *in vitro*, however, there is considerable potential for interaction with other molecules *in vivo*.

L-arginine site

NO itself inhibits NOS activity at the L-arginine binding site. N(G)-monomethyl-L-arginine (L-NMMA) and L-NIO work in a similar fashion to NO, with the gradual decrease in NO production as the enzyme becomes inactivated due to alkylation of haem by substituents of the inhibitor (Raman et al 2001).

N-3-aminoethyl-benzyl-acetamidine hydrochloride (1400W) is a slow, tight binding and highly selective iNOS inhibitor that requires NADPH, with L-arginine acting as a competitive inhibitor of 1400W binding (Garvey et al 1997). Crystal structure binding of 1400W to eNOS revealed that the inhibitor binds haem, suggesting it is likely to be iNOS selective partially due to higher iNOS turnover, when compared with e and nNOS, leading to peroxide production and irreversible haem damage (Li et al 2001). Recent work has, however, revealed the binding of 1400W to both the active site and to isoform specific residues outside the active site, with conformational flexibility of the active site allowing structural changes when bound to the inhibitor (Fedorov et al 2003). Despite good penetration into tissues and cells, and high specificity, there is toxicity associated with this compound that is likely to prevent its use as a therapeutic agent in human conditions (Garvey et al 1997).

The GW compounds (3-[[2-(ethanimidoylamino)ethyl]sulphonyl]-L-alanine) are hetero-substituted analogues and homologues of L-NIL, with similar potency to L-NIL but significantly increased iNOS isoform selectivity. The activity of these compounds is maintained in intact cells and tissues, with no evidence of acute toxicity (Cuzzocrea et al 2002; Young et al 2000).

Tetrahydrobiopterin (BH_4) site

Some pterin analogues such as 4-amino-BH_4 and BH_2 exist, as well as other non-analogue inhibitors including 7-nitroindazole (7NI) and 3-Br-7-nitroindazole (7NIBr) (Bommel et al 1998). 7-NI (Alderton et al 2001) and 7NIBr (Bland-Ward and Moore 1995) inhibit all three NOS isoforms in vitro. High level nNOS specificity is seen *in vivo*, as monitored by a lack of changes in blood pressure (Handy and Moore 1998). It is thought that different cell-type specific uptake pathways may play a role in this selectivity, conferring neuroprotective properties on mouse models of stroke (Nanri et al 1998) and Parkinsons disease (Zhang et al 2000). Analysis of the crystal structure of the catalytic haem domain of eNOS bound to 7NIBr by Raman et al (2001) showed inhibitor binding causes conformational change in the enzyme, causing elimination of the BH_4-haem interaction.

Inhibitors of NOS dimerisation

The pyrimidine imidazoles are potent and selective inhibitors of iNOS dimerization, identified from a library of compounds using a cell based assay with a specific radiolabel for iNOS in its monomeric form (McMillan et al 2000). These were found to be 1000 fold more potent than mechanism based inhibitors such as 1400W, and 1000 fold more selective for iNOS compared to eNOS *in vitro* with activity *in vivo*, suggesting therapeutic potential. Crystal structure analysis of the enzyme bound to the inhibitor revealed that the molecule acts by allosterically disrupting the protein-protein interactions at the dimer interface.

The determination of the selectivity of NOS inhibitors requires the use of isolated NOS enzymes. Previous studies have identified isoform selectivity through *in vivo* physiological effects which can lead to inappropriate classification of inhibitors. The iNOS inhibitor AR-C102222 was identified as a molecule that had shown a 50% inhibition (IC_{50}) of isolated iNOS enzyme activity at 0.037μM, eNOS IC_{50} of >100 μM and nNOS IC_{50} of 1μM. The activity was also assessed in the human adenocarcinoma cell line DLD-1 with an IC_{50} of 0.9μM and an in vivo IC_{50} in rat of 3μmol/kg (Tinker et al 2003).

In vitro analysis of the inhibitor AR-C102222 shows strong iNOS selectivity and good cell permeability (Tinker et al 2003). The inhibitor is a spirocyclic quinazolinamine. This molecule is not an analogue of arginine, but the requirement for the use of a medium containing low arginine suggests that it acts through competition with the enzyme substrate. Our study showed that the inhibitor does not inhibit iNOS mRNA or protein levels following CYT stimulation of OA HAC. However, a dose dependent decrease in the level of nitrite detected is seen, with 10^{-4} and 10^{-5}M completely inhibiting nitrite while10^{-6}M shows incomplete inhibition. This suggests the inhibitor functions as an inhibitor of enzyme activity rather than through alteration of mRNA or protein levels. This is most likely to be through its competition with L-arginine for the enzyme substrate binding site. It is possible, however, that some other interaction with the iNOS protein may cause structural changes that could alter enzyme activity.

4.4 - Conclusion

Understanding the mechanisms involved in mechanical signalling in cartilage and chondrocytes is greatly complicated by the heterogeneity of cartilage and cells, and the plethora of factors that have the potential to influence signals being sensed by each chondrocyte *in vivo*. The present situation is also complicated by the fact that each group of investigators uses different strain frequencies and loads as well as different cells and cell culture conditions to study the effects of strain. Despite these drawbacks there is a general trend which demonstrates that cyclic strain at low loads and frequencies appears to be beneficial and anti-inflammatory, as seen in our experiments where MS inhibits CYT induction of iNOS. By contrast, static strains, as well as higher loads and frequencies, are catabolic for cartilage matrix homeostasis. It is therefore necessary to understand the forces and loads experienced by these cells *in vivo* in order to allow appropriate study of the intracellular signalling mechanisms in *in vitro* models. The exact nature of the strain experienced by these cells is complicated by the fact that, aside from the cyclic tensile strain they also experience some shear stress due to fluid flow, and this is known to have effects on chondrocytes including altering matrix synthesis (Das et al 1997). The relative lack of pericellular matrix produced by these cells in monolayer culture means they do not have the appropriate matrix that is seen in cartilage itself, so all strains will be greater than the *in vivo* situation (Urban 1994).

However, despite the limitations of the experimental system it provides a model through which the signalling pathways activated by mechanical strain can be studied. This series of experiments has shown that strain is not working through a β1 integrin or an IL-4 mediated pathway to decrease the levels of iNOS mRNA, protein and nitrite detected following proinflammatory cytokine stimulation. The potential interactions through the MAPK and PKC signalling, actions on transcription factor synthesis and activation as well as the presence of mechano-responsive elements on gene promoters are all factors that need further study in order to fully understand how interactions could be leading to changes in gene expression.

Summary of findings

- Primary osteoarthritic human articular chondrocytes produce e, n and iNOS mRNA, however no e or nNOS protein is detected.

- Primary osteoarthritic human articular chondrocytes produce iNOS mRNA, protein and nitrite following stimulation with a proinflammatory cytokine cocktail or IL-1β alone.

- The application of mechanical stimulation (0.33Hz, 30,000μstrain) reduces the level of iNOS seen following CYT or IL-1β stimulation. This decrease occurs through a β1 integrin and IL-4 independent mechanism.

- A novel iNOS inhibitor, AR-C102222, inhibits iNOS enzyme activity without affecting its mRNA or protein expression.

Chapter 5 – Effects of cyclical mechanical stimulation, CYT and IL-1β on aggrecan mRNA levels in primary OA HAC – is there a role for NO?

Aims

- Investigate the effects of mechanical stimulation and nitric oxide on proteoglycan levels in primary osteoarthritic human articular chondrocytes

5.1 – Introduction

Aggrecan mRNA levels were detected in primary osteoarthritic (OA) human articular chondrocytes (HAC). Taqman real-time PCR was used to measure aggrecan mRNA levels using two separate primer/probe sets that have both been designed to detect human aggrecan mRNA. These are described as primer/probe sets 1 and 2, with set 1 primarily used and set 2 used to confirm results in several experimental series.

In the previous chapter the level of inducible nitric oxide synthase (iNOS) mRNA, protein and NOS enzyme activity was determined in primary OA HAC. Here, nitrite levels were detected by the Griess assay as a measure of nitric oxide (NO) release into the medium by the NO donor SNAP following proinflammatory cytokine cocktail stimulation (CYT).

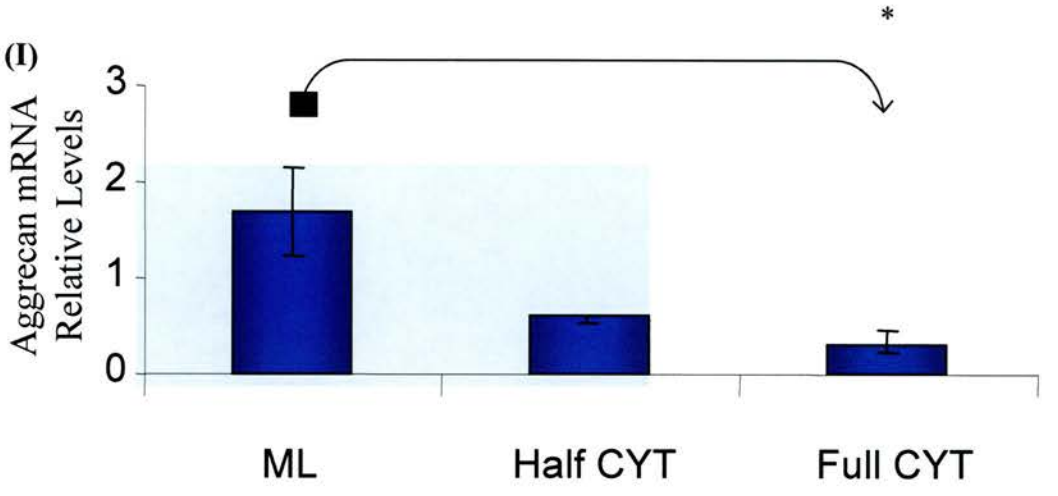
Primary OA HAC were initially stimulated with either CYT or IL-1β alone and the effect of dose on aggrecan mRNA expression was evaluated. The effect of cyclical mechanical stimulation at 0.33Hz and 30,000μstrain (MS) on both CYT and IL-1β stimulated aggrecan mRNA levels was then studied. The method by which MS may be interacting with CYT and IL-1β stimulated aggrecan mRNA levels was investigated using IL-4 neutralising antibodies and a β1 integrin function blocking antibody. The effect of NO on aggrecan mRNA levels was studied using a novel iNOS inhibitor, AR-C102222, and an NO donor, SNAP.

5.2 - Results

5.2.1 – Effects of CYT stimulation on aggrecan mRNA levels

5.2.1a - Aggrecan mRNA levels in primary OA HAC

Aggrecan mRNA was detected in unstimulated primary human OA HAC from cycle 25 using primer/probe set 1. Aggrecan mRNA levels were decreased 65% and 80% at 12 hours following incubation with half and full concentration of CYT respectively ($p=0.1$ and $p=0.047$ respectively) (**Figure 5.2.1**).



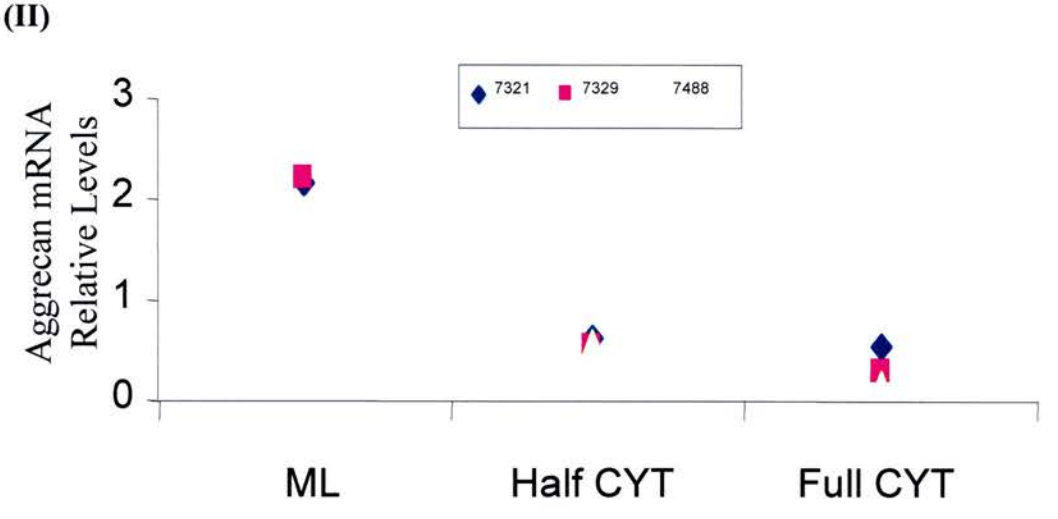


Figure 5.2.1 - Basal (unstimulated) and cytokine cocktail stimulated aggrecan mRNA levels (12hr). (I) = Pooled values±SEM. (II) = Individual experimental values. ML = Unstimulated control. Half CYT = Half concentration of cytokine cocktail. Full CYT = Cytokine cocktail stimulation. (n=3). *$p<0.05$.

5.2.1b – Effects of CYT and MS on aggrecan mRNA levels

Aggrecan mRNA levels were detected at 4 and 12 hours following CYT and MS using primer/probe set 1.

MS decreased aggrecan mRNA levels at 4 hours, 37% below those seen in the unstimulated control ($p=0.16$) (**Figure 5.2.2**). MS did not alter the level of aggrecan mRNA detected from those seen in the unstimulated control at 12 hours.

Lower aggrecan mRNA levels were detected following CYT stimulation than in the unstimulated control: 41% lower at 4 hours and 37% lower at 12 hours ($p=0.09$ and $p=0.04$ respectively).

Simultaneous stimulation with CYT and MS decreased aggrecan mRNA levels compared to unstimulated controls by 37% at 4 hours and 50% at 12 hours ($p=0.11$ and $p<0.001$ respectively). The level of aggrecan mRNA following CYT plus MS was not altered when compared to CYT alone at both 4 and 12 hours.

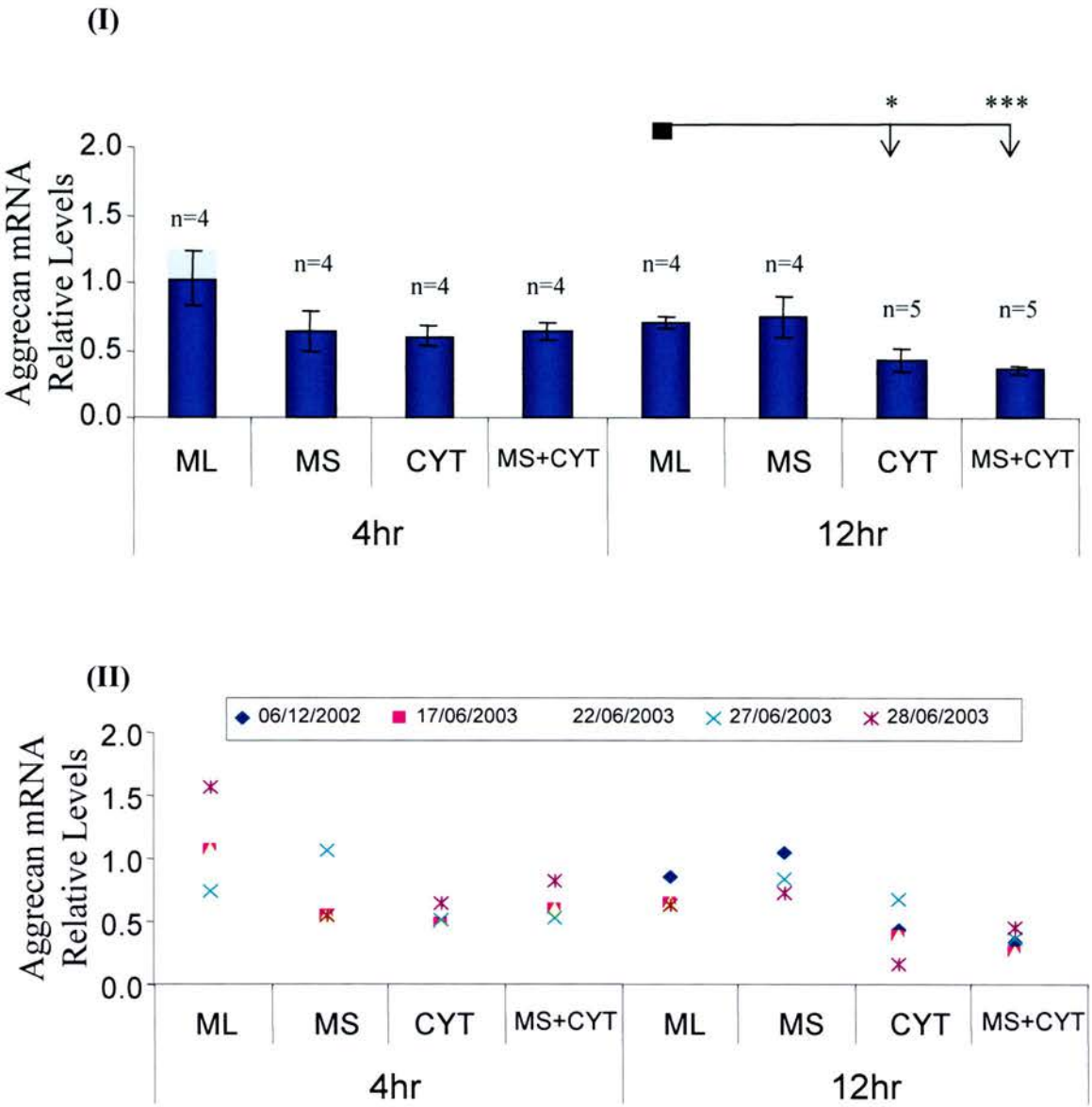


Figure 5.2.2 – Aggrecan mRNA levels following CYT and MS. (I) = Pooled iNOS mRNA levels±SEM. (II) = Individual experimental values. ML = Unstimulated control. MS = Mechanical stimulation. CYT = Cytokine cocktail stimulation. CYT+MS = Both cytokine cocktail and mechanical stimulation. *$p<0.05$ ***$p<0.001$.

5.2.1c – Effects of CYT, MS and an IL-4 neutralising antibody on aggrecan mRNA levels

Aggrecan mRNA levels following CYT stimulation in combination with MS and a monoclonal IL-4 neutralising antibody (1μg/ml) were measured by Taqman real-time PCR using two separate human aggrecan primer/probe sets (primer/probe sets 1 and 2).

There was little alteration in aggrecan mRNA levels detected at 4 hours following any of the stimuli using either primer/probe set 1 or 2 (**Figures 5.2.3 and 5.2.4**). The exception to this was where a 1.6 fold and 1.7 fold increase in aggrecan mRNA levels above unstimulated controls were seen following simultaneous CYT, MS and IL-4 neutralising antibody stimulation using primer/probe sets 1 and 2 respectively. However this increase was not statistically significant.

At 12 hours following both CYT and CYT in combination with the IL-4 neutralising antibody no change was seen in aggrecan mRNA levels using either primer/probe set. CYT in combination with MS at 12 hours decreased aggrecan mRNA levels by 44% and 41% compared to unstimulated controls using primer/probe sets 1 and 2 respectively ($p=0.02$ and $p=0.07$ respectively). The simultaneous application of CYT, MS and IL-4 neutralising antibody did not alter aggrecan mRNA levels from the levels seen following CYT plus MS using primer/probe set 1 or 2.

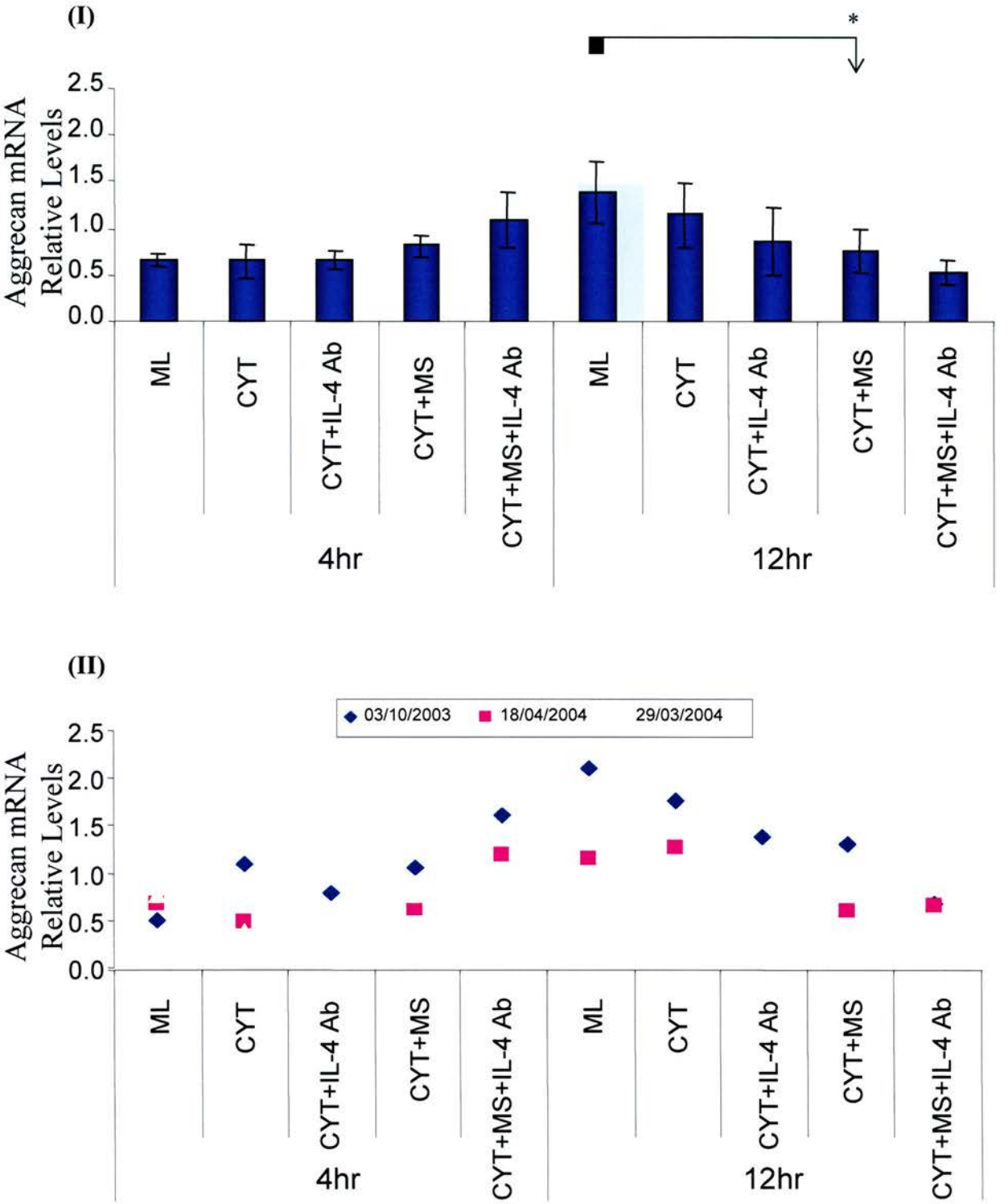


Figure 5.2.3 – Aggrecan mRNA levels detected with primer/probe set 1 following incubation with a monoclonal IL-4 neutralising antibody.
(I) = Pooled data±SEM. (II) = Individual experimental values.
ML = Unstimulated control. CYT = Cytokine cocktail stimulation. IL-4 Ab = Monoclonal IL-4 neutralising antibody at 1µg/ml. MS = Mechanical stimulation. (n=3). *$p<0.05$ (paired t test used).

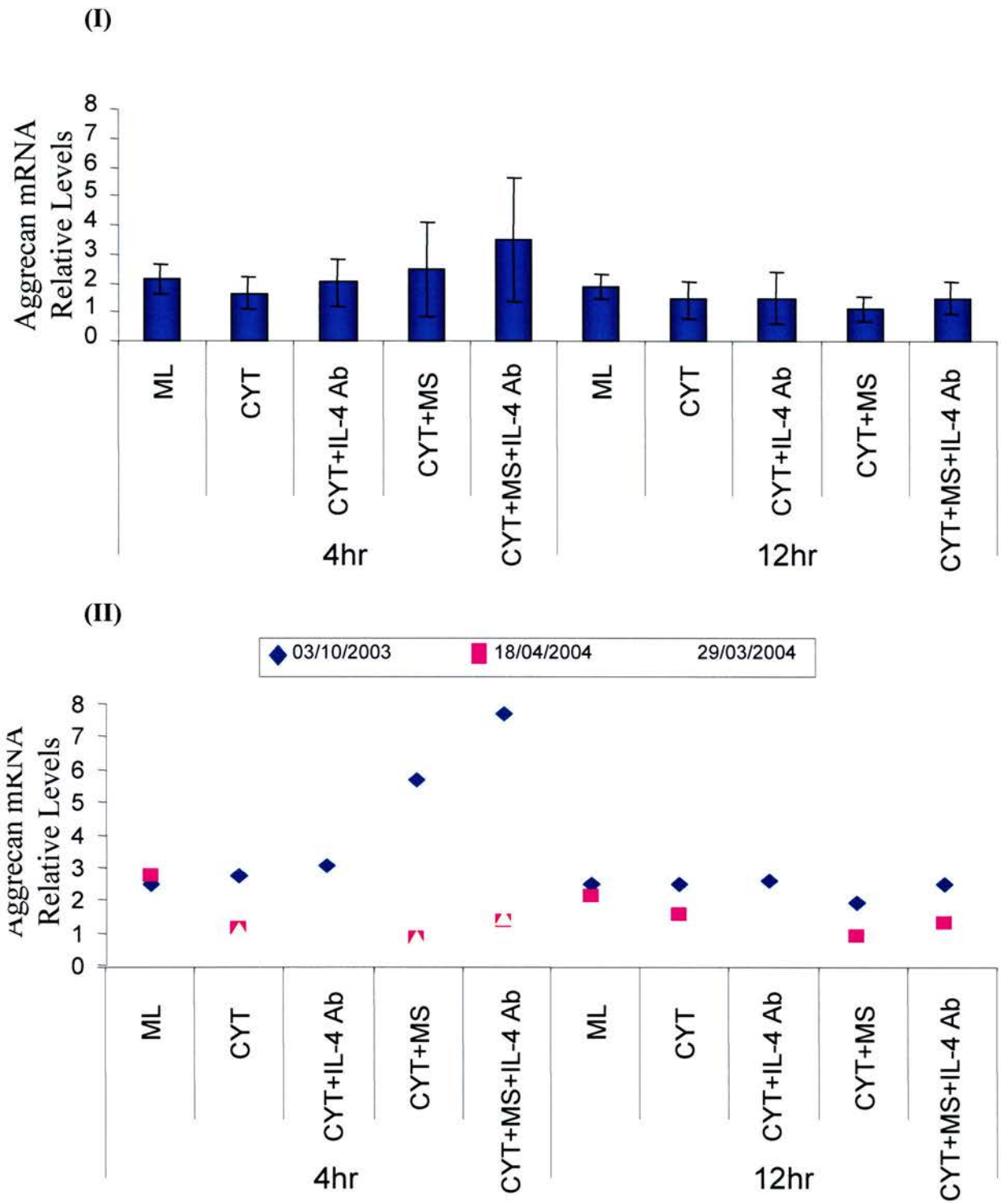


Figure 5.2.4 - Aggrecan mRNA levels detected with primer/probe set 2 following incubation with a monoclonal IL-4 neutralising antibody.
(I) = Pooled data±SEM. (II) = Individual experimental values.
ML = Unstimulated control. CYT = Cytokine cocktail stimulation. IL-4 Ab = Monoclonal IL-4 neutralising antibody at 1µg/ml. MS = Mechanical stimulation. (n=3). (paired t test used).

5.2.1d – Effects of CYT, MS and a β1 integrin function blocking antibody on aggrecan mRNA levels

Aggrecan mRNA levels following CYT stimulation in combination with MS and a β1 integrin function blocking antibody (1μg/ml) were measured by Taqman real-time PCR using the two separate human aggrecan primer/probe sets.

The addition of the β1 integrin function blocking antibody (1μg/ml) alone was carried out in a single experiment (10/2/2004). Aggrecan mRNA increased by 3.1 and 2.8 fold at 4 hours and 2 and 1.5 fold and at 12 hours using primer/probe sets 1 and 2 respectively (**Figures 5.2.5 and 5.2.6**).

At 4 hours CYT and CYT plus β1 integrin function blocking antibody did not appear to alter the aggrecan mRNA levels compared to the unstimulated control using either primer/probe set 1 or 2. The analysis of single experiments, however, revealed that the 8/3/04 sample shows a 3.8 and a 1.9 fold increase in aggrecan mRNA following CYT stimulation using primer/probe sets 1 and 2 respectively, a response which was lost at 12 hours. The application of CYT+MS appeared to elevate the aggrecan mRNA levels by 2.7 and 1.5 fold at 4 hours using primer/probe sets 1 and 2 respectively, but this was not statistically significant. Analysis of the individual experimental values, however, revealed that these values were heavily influenced by the sample from 22/03/04.

At 12 hours CYT and CYT plus MS the aggrecan mRNA levels were decreased by 60% and 58% respectively when compared to unstimulated controls and detected using primer/probe set 1 ($p=0.07$ and $p=0.02$ respectively). Primer/probe set 2, however, did not show such a clear decrease in aggrecan mRNA: 23% and 29% following CYT and CYT plus MS respectively ($p=0.18$ and $p=0.11$). The simultaneous application of β1 integrin function blocking antibody did not alter the level of aggrecan mRNA in combination with CYT or CYT plus MS using either primer/probe set. The use of both primer/probe sets revealed that CYT in combination with the β1 integrin function blocking antibody induced a higher level of aggrecan mRNA than CYT alone at 12 hours in the sample from 22/03/04.

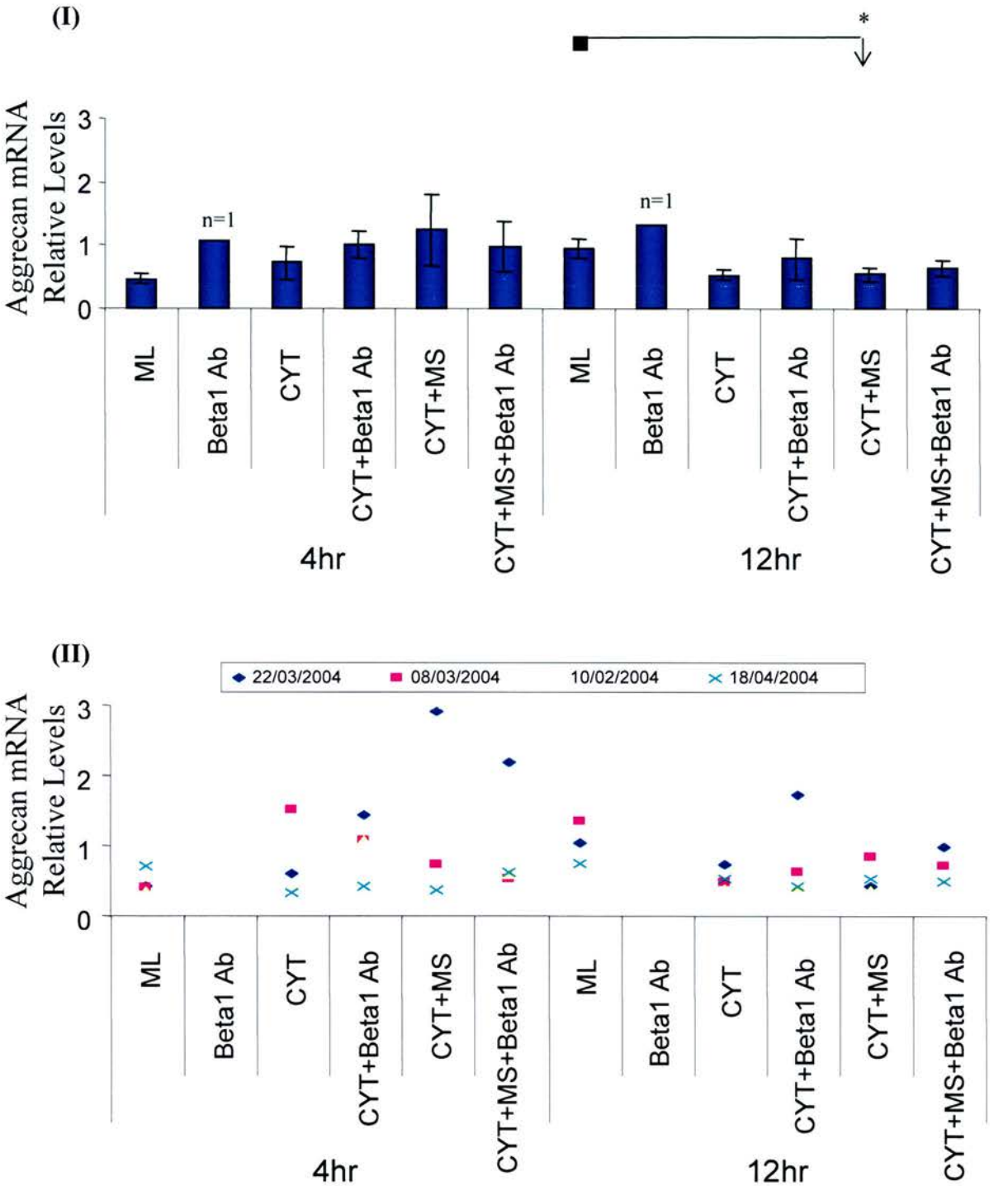


Figure 5.2.5 – Aggrecan mRNA levels detected with primer/probe set 1 following incubation with a β1 integrin blocking antibody. (I) = Pooled data±SEM. (II) = Individual experimentalvalues. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. Beta1 Ab = β1 integrin function blocking antibody at 1μg/ml. MS = Mechanical stimulation. (n=4). *$p<0.05$ (paired t test used)

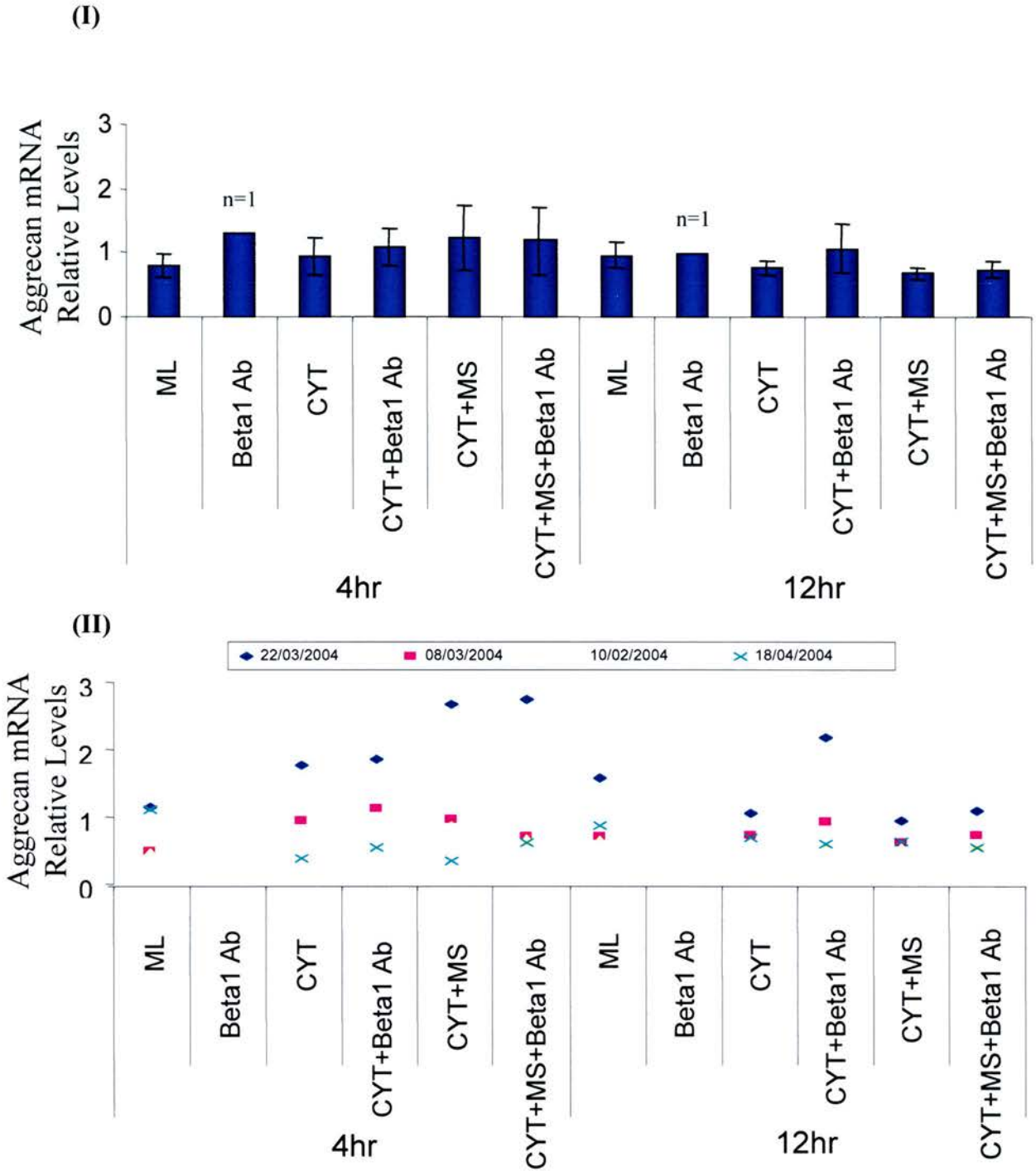


Figure 5.2.6 – Aggrecan mRNA levels detected with primer/probe set 2 following incubation with a β1 integrin blocking antibody. (I) = Pooled data±SEM. (II) = Individual experimental values. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. Beta1 Ab = β1 integrin function blocking antibody at 1μg/ml. MS = Mechanical stimulation. (n=4). (paired t test used)

5.2.1e – Effects of CYT, MS, IL-4 neutralising antibody and β1 integrin function blocking antibody on pooled aggrecan mRNA values

Aggrecan mRNA levels were detected using primer/probe set 1.

The level of aggrecan mRNA was decreased compared to the unstimulated control at 4 hours following MS, CYT alone and CYT in combination with MS by 44%, 29% and 23% respectively ($p=0.19$, $p=0.09$ and $p=0.38$) (**Figure 5.2.7**).

At 12 hours aggrecan mRNA levels were 41%, 55% and 58% lower than the unstimulated control following MS, CYT alone and CYT in combination with MS respectively ($p=0.21$, $p=0.0001$ and $p<0.0001$).

The aggrecan mRNA levels following CYT or CYT plus MS were not altered following 4 or 12 hour incubation with a monoclonal IL-4 neutralising antibody (1μg/ml) or a β1 integrin function blocking antibody (1μg/ml).

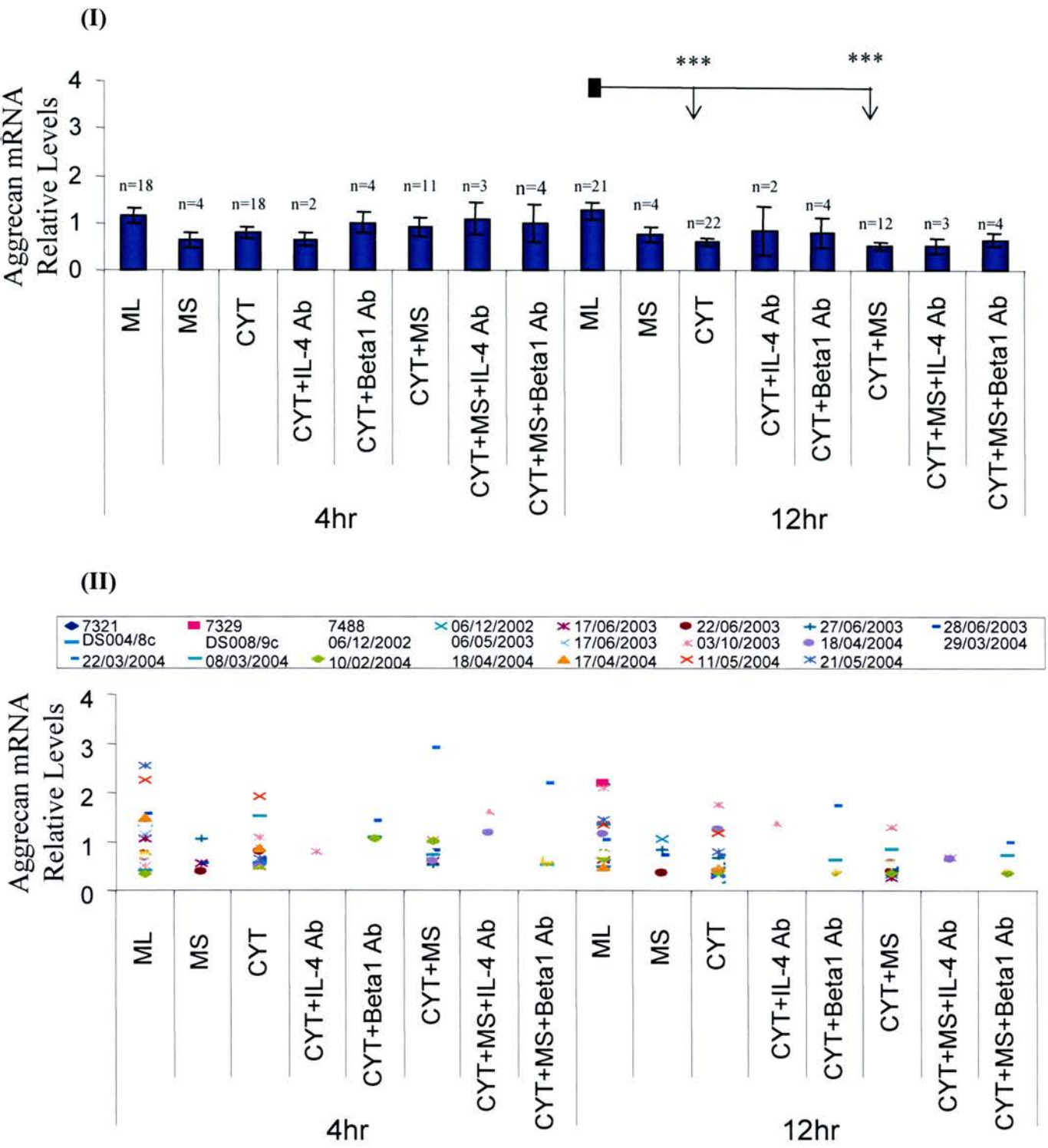


Figure 5.2.7 – Pooled aggrecan mRNA levels detected with primer/probe set 1 following incubation with an IL-4 neutralising antibody or a β1 integrin blocking antibody. (I) = Pooled data±SEM. (II) = Individual experimental values. ML = Unstimulated control. CYT = Cytokine cocktail stimulation. Beta1 Ab = β1 integrin function blocking antibody at 1μg/ml. IL-4 Ab = IL-4 neutralising antibody at 1μg/ml. MS = Mechanical stimulation. ***$p<0.001$.

5.2.2 – Effects of IL-1β on aggrecan mRNA levels

5.2.2a – Effects of IL-1β at a range of concentrations on aggrecan mRNA levels

Primary OA HAC were incubated with IL-1β at a range of concentrations and the effect on aggrecan mRNA levels was evaluated using both primer/probe sets 1 and 2.

Aggrecan mRNA levels were not altered from unstimulated control levels by IL-1β from 10 to 2500pg/ml at 4 hours using either primer probe set (**Figures 5.2.8 and 5.2.9**). The exception to this was 100pg/ml IL-1β stimulation of the sample from 18/04/04, which showed a 2.1 and 1.2 fold increase in aggrecan mRNA levels compared to the unstimulated control using primer/probes 1 and 2 respectively.

At 12 hours aggrecan mRNA levels were lower than unstimulated levels following IL-1β incubation from 10pg/ml to 2500pg/ml. Primer probe set 1 showed levels lower than unstimulated controls of 33%, 47%, 58% and 64% for 10, 100, 500 and 2500pg/ml IL-1β respectively, however these were not statistically significant. Primer/probe set 2 showed levels lower than unstimulated controls of 41%, 41%, 52% and 52% for 10, 100, 500 and 2500pg/ml respectively, however these were not statistically significant.

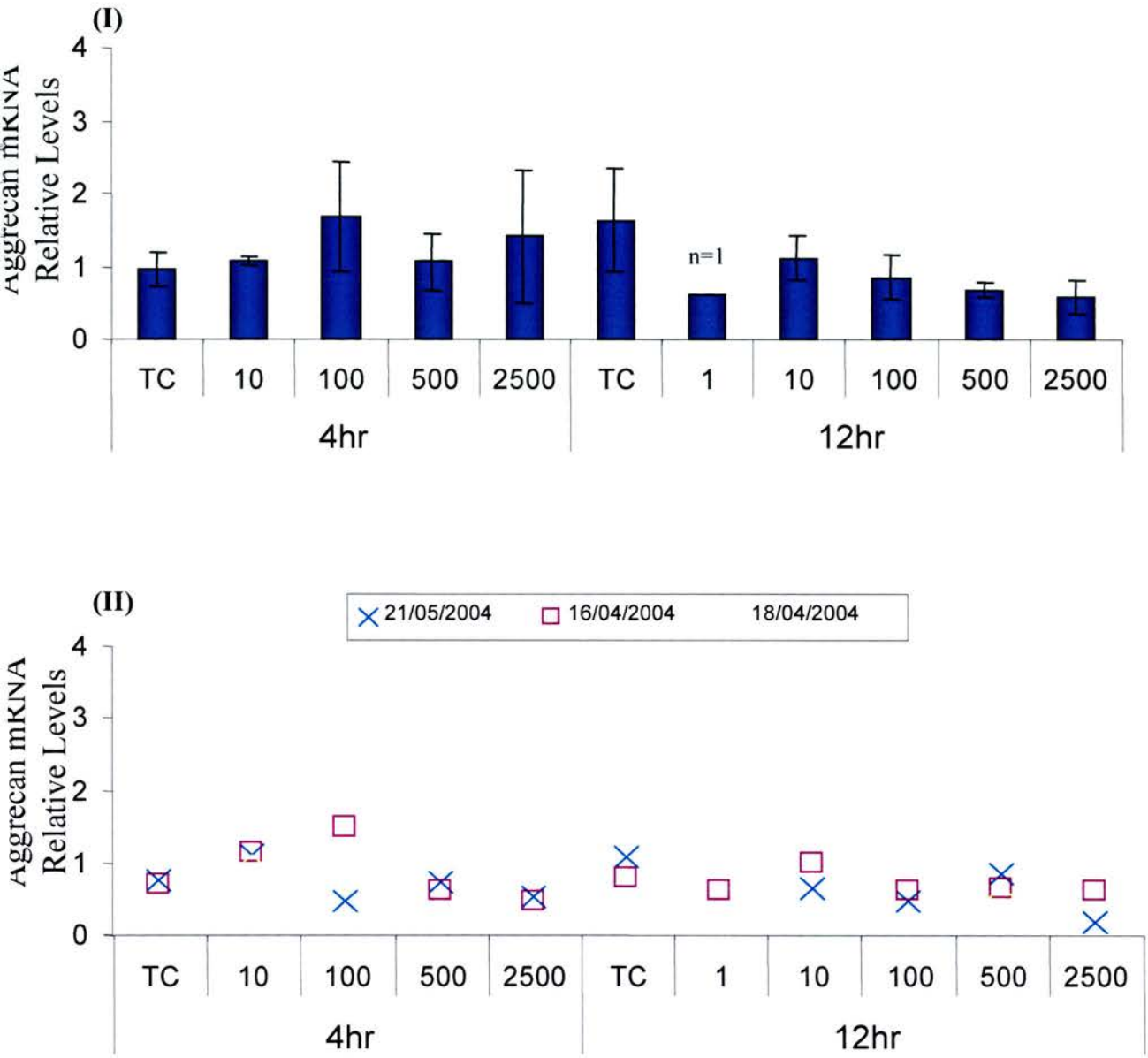


Figure 5.2.8 – Aggrecan mRNA levels detected with primer/probe set 1 following IL-1β stimulation. IL-1β values in pg/ml. TC = Unstimulated control. (I) = Pooled data±SEM. (II) = Individual experimental values. (n=3)

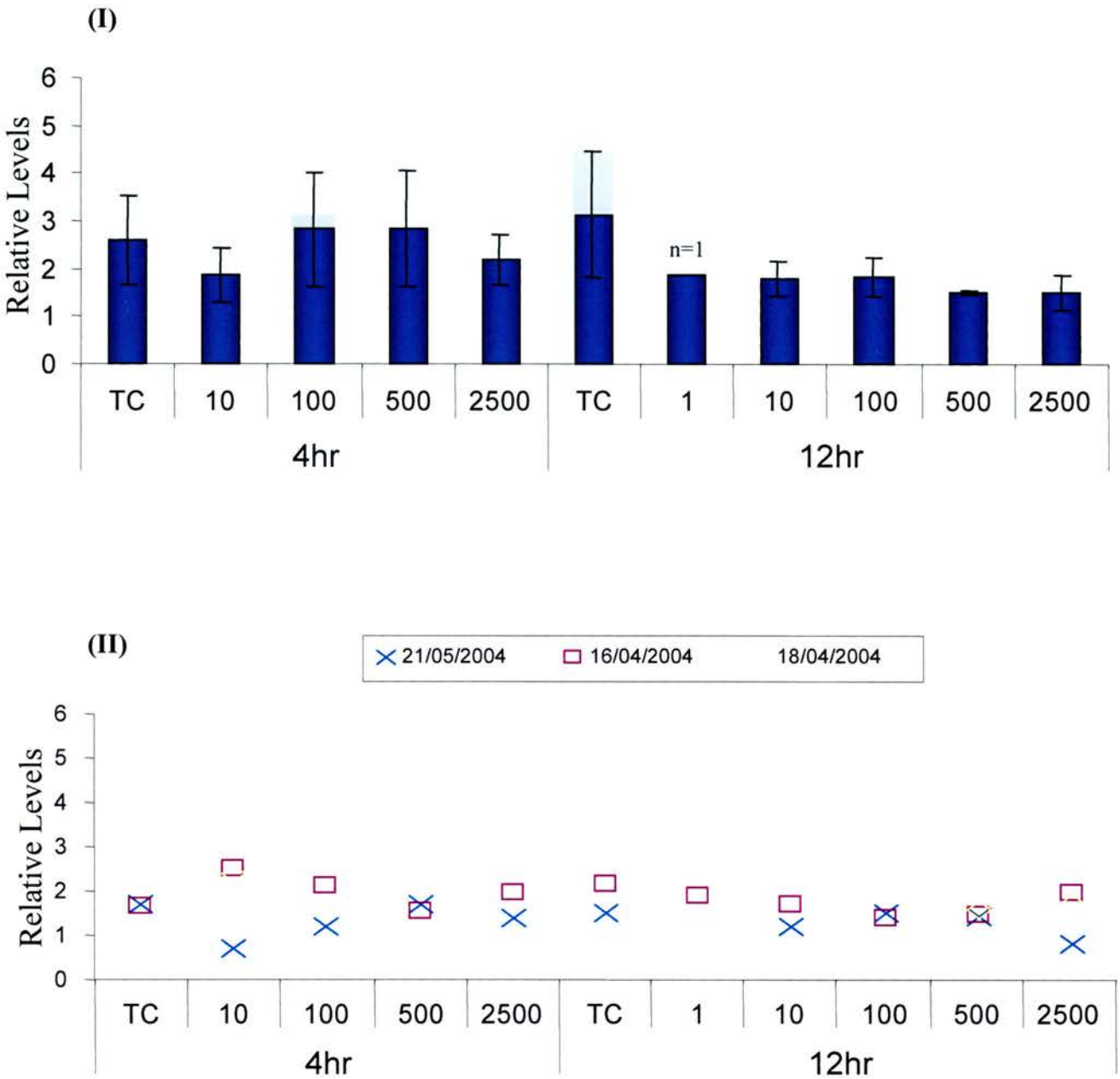


Figure 5.2.9 – Aggrecan mRNA levels detected with primer/probe set 2 following IL-1β stimulation. TC = Unstimulated control. IL-1β values in pg/ml. (I) = Pooled data±SEM. (II) = Individual experimental values. (n=3)

5.2.2b - Effects of IL-1β, MS, IL-4 neutralising antibody and β1 integrin function blocking antibody on aggrecan mRNA levels

The effect of IL-1β stimulation (500pg/ml) on aggrecan mRNA levels following incubation with MS, a monoclonal IL-4 neutralising antibody (1μg/ml) or a β1 integrin function blocking antibody (1μg/ml) was evaluated using both primer/probe sets 1 and 2 in primary OA HAC.

Aggrecan mRNA levels were 55% and 23% lower at 4 hours and 64% and 33% lower at 12 hours than the unstimulated control following IL-1β stimulation detected with primer/probe sets 1 and 2 respectively (p=0.38, p=0.09, p=0.04 and p=0.09) (**Figures 5.2.10 and 5.2.11**).

Primer/Probe set 1

Primer/probe set 1 (**Figure 5.2.10**) detected aggrecan mRNA at 17% and 44% lower levels than in unstimulated controls following IL-1β plus MS at 4 and 12 hours respectively (p=0.78 and p=0.024). The aggrecan mRNA levels following IL-1β plus MS were 1.9 and 1.6 fold higher than following IL-1β stimulation alone at 4 and 12 hours respectively (p=0.01 and p=0.24). Incubation with IL-1β, MS and a β1 integrin function blocking antibody (1μg/ml) showed aggrecan mRNA levels that are similar to those following IL-1β stimulation alone at 4 and 12 hours. The aggrecan mRNA levels following IL-1β, MS and a β1 integrin function blocking antibody (1μg/ml) were 55% and 62% lower than those seen following IL-1β plus MS at 4 and 12 hours, although these values did not reach statistical significance. Incubation with a monoclonal IL-4 neutralising antibody (1μg/ml) did not alter the aggrecan mRNA levels when in combination with IL-1β or IL-1β plus MS compared to those following IL-1β or IL-1β plus MS alone at 4 or 12 hours. Analysis of the individual experimental values, however, revealed that the monoclonal IL-4 neutralising antibody was associated with 1.7 fold higher aggrecan mRNA levels in two samples when in combination with IL-1β and IL-1β plus MS than following IL-1β or IL-1β plus MS without antibody at 4 hours.

Primer/Probe set 2

Primer/probe set 2 (**Figure 5.2.11**) showed little change in aggrecan mRNA levels following any of the stimuli at 4 hours. Incubation with IL-1β decreased aggrecan mRNA levels, although this was not statistically significant ($p=0.09$). Incubation with IL-1β plus MS did not cause altered aggrecan mRNA levels compared to IL-1β alone. Incubation with either a monoclonal IL-4 neutralising antibody or a β1 integrin function blocking antibody did not appear to influence aggrecan mRNA levels.

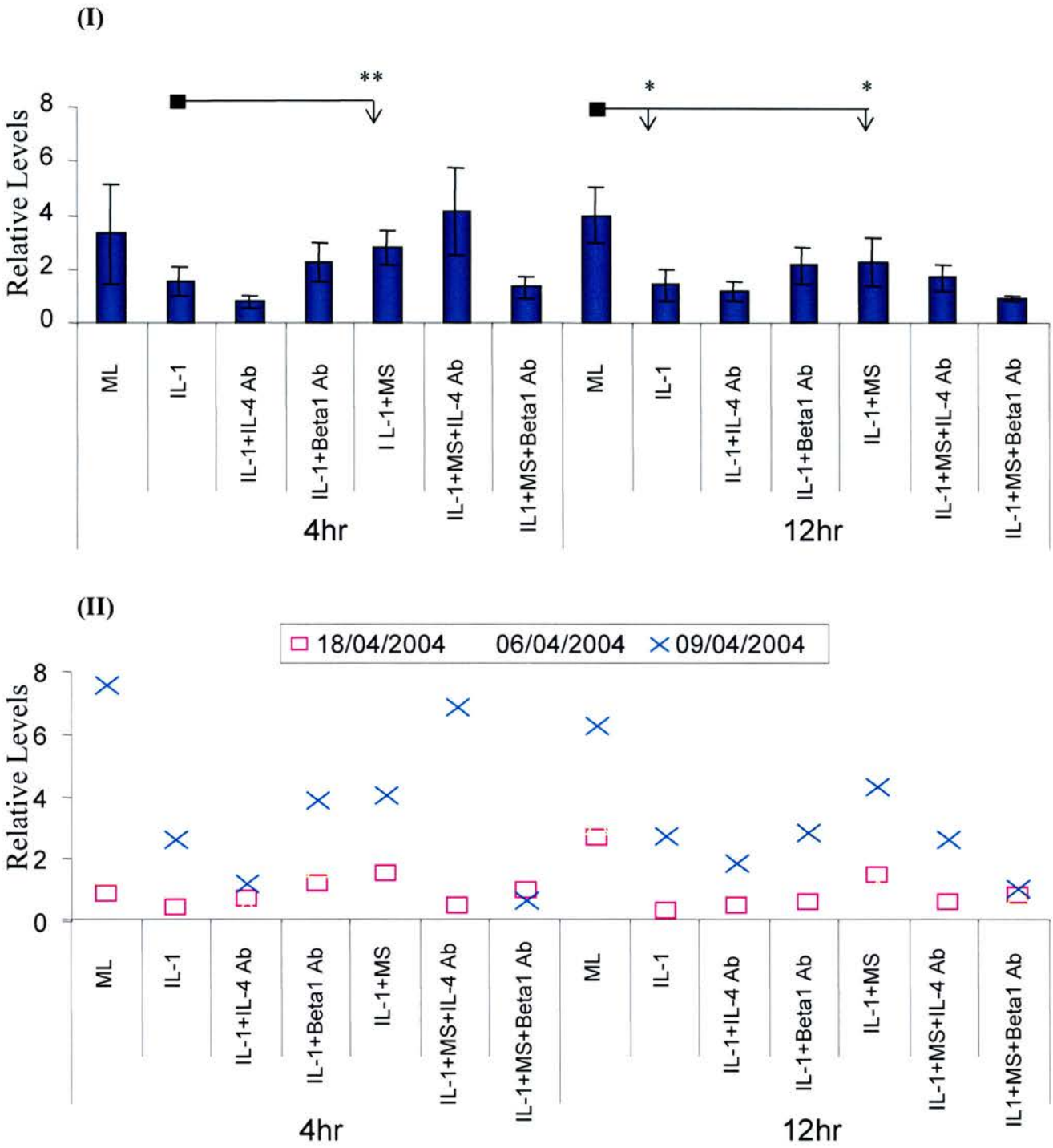


Figure 5.2.10 – Aggrecan mRNA levels detected with primer/probe set 1 following incubation with IL-1β, a monoclonal IL-4 neutralising antibody or a β1 integrin function blocking antibody. *$p<0.05$ **$p<0.01$ (paired t test used).
(I) = Pooled data±SEM. (II) = Individual experimental values.
ML = Unstimulated control. IL-1 = IL-1β stimulation (500pg/ml). Beta1 Ab = β1 integrin function blocking antibody (1μg/ml). IL-4 Ab = IL-4 neutralising antibody (1μg/ml). MS = Mechanical stimulation. (n=3)

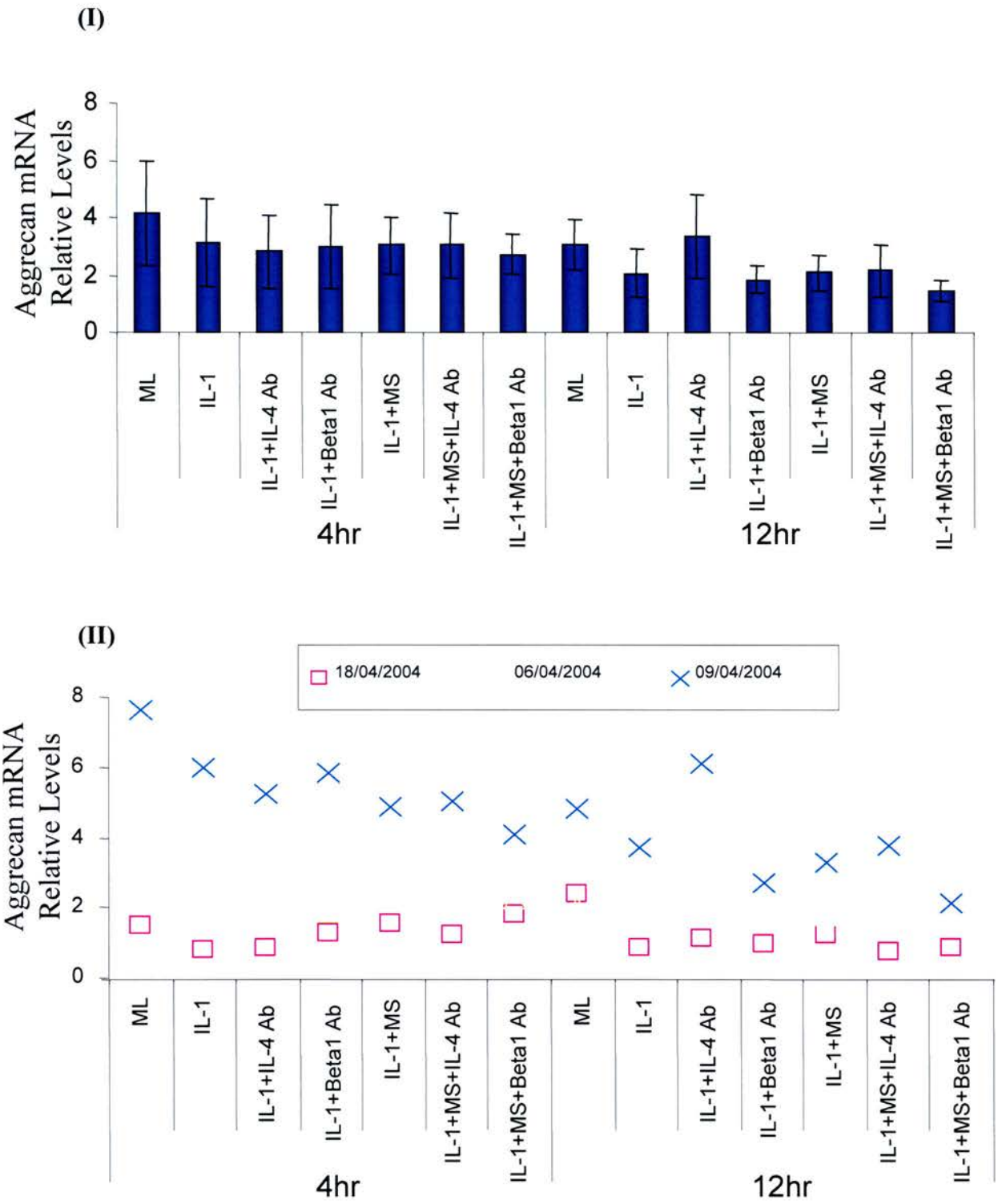


Figure 5.2.11 – Aggrecan mRNA levels detected with primer/probe set 2 following incubation with IL-1β, a monoclonal IL-4 neutralising antibody or a β1 integrin function blocking antibody.
(I) = Pooled data±SEM. (II) = Individual experimental values.
ML = Unstimulated control. IL-1 = IL-1β stimulation (500pg/ml). Beta1 Ab = β1 integrin function blocking antibody (1μg/ml). IL-4 Ab = monoclonal IL-4 neutralising antibody (1μg/ml). MS = Mechanical stimulation. (n=3)

5.2.3 – Effects of Nitric Oxide (NO) on aggrecan mRNA levels

5.2.3a – Effects of the iNOS Inhibitor AR-C102222 on aggrecan mRNA levels

Aggrecan mRNA levels were detected in primary OA HAC with both primer probe sets 1 (**Figure 5.2.12**) and 2 (**Figure 5.2.13**) following incubation with CYT and 10^{-4}, 10^{-5} and 10^{-6}M of the iNOS inhibitor AR-C102222.

Primer/Probe set 1

Aggrecan mRNA levels detected with primer/probe set 1 following incubation with inhibitor alone at 10^{-4}, 10^{-5} and 10^{-6}M were 23%, 55% and 42% lower than unstimulated control levels at 4 hours respectively (p=0.32, p=0.04 and p=0.05) (**Figure 5.2.12**). The level of aggrecan mRNA following incubation with the inhibitor at 10^{-4}, 10^{-5} and 10^{-6}M was not different from unstimulated controls at 12 hours. Aggrecan mRNA levels were 44% and 23% lower than unstimulated controls following CYT at 4 and 12 hours respectively (p=0.18 and p=0.29). The inhibitor at 10^{-4}, 10^{-5} and 10^{-6}M did not influence aggrecan mRNA levels in combination with CYT at 12 hours.

Primer/Probe set 2

The inhibitor at 10^{-4}, 10^{-5} and 10^{-6}M did not change aggrecan mRNA levels from unstimulated controls at 4 or 12 hours using primer/probe set 2 (**Figure 5.2.13**). Aggrecan mRNA levels were 29% and 33% lower at 4 and 12 hours respectively following CYT than in unstimulated controls (p=0.3 and p=0.02). The inhibitor at 10^{-4}, 10^{-5} and 10^{-6}M in combination with CYT does not alter aggrecan mRNA levels following CYT alone.

Pooled values

The levels detected using primer/probe set 1 were pooled with an earlier study to allow greater confidence when calculating significance (**Figure 5.2.14**). These showed that aggrecan mRNA levels at 4 hours were 33%, 37% and 50% lower following incubation with the inhibitor at 10^{-4}, 10^{-5} and 10^{-6}M respectively (p=0.1, p=0.09 and p=0.012). The inhibitor at 10^{-4}, 10^{-5} and 10^{-6}M also showed 33%, 23% and 55% lower aggrecan mRNA levels at 12 hours compared to unstimulated

controls, although statistical significance is not reached. The level of aggrecan mRNA was 44% and 62% lower than unstimulated controls following CYT stimulation at 4 and 12 hours ($p=0.028$ and $p=0.07$). The inhibitor at 10^{-4}, 10^{-5} and 10^{-6}M did not alter aggrecan mRNA levels when in combination with CYT compared to CYT alone at 4 or 12 hours. The possible exception was 10^{-5}M inhibitor in combination with CYT, which showed a 1.3 fold increase in aggrecan mRNA levels at 4 hours compared to CYT alone, although this was not statistically significant.

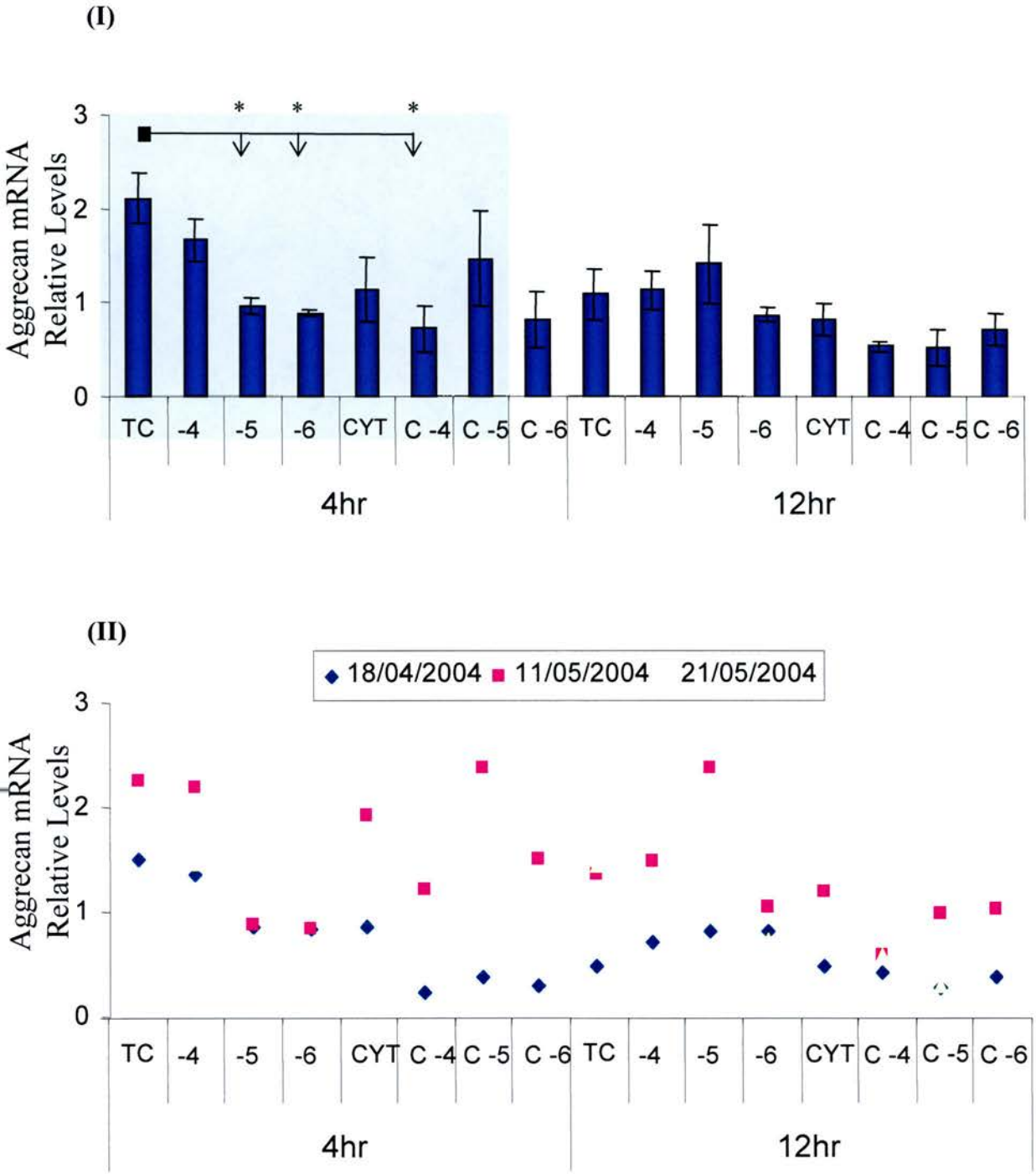


Figure 5.2.12 - Aggrecan mRNA levels detected with primer/probe set 1 following incubation with CYT and the iNOS inhibitor AR-C102222.
(I) = Pooled data±SEM. (II) = Individual experimental values.
TC = Unstimulated control. CYT and C = Cytokine cocktail stimulation. $-4 = 10^{-4}$M Inhibitor. $-5 = 10^{-5}$M Inhibitor. $-6 = 10^{-6}$M Inhibitor. (n=3). *$p<0.05$ (paired t test used)

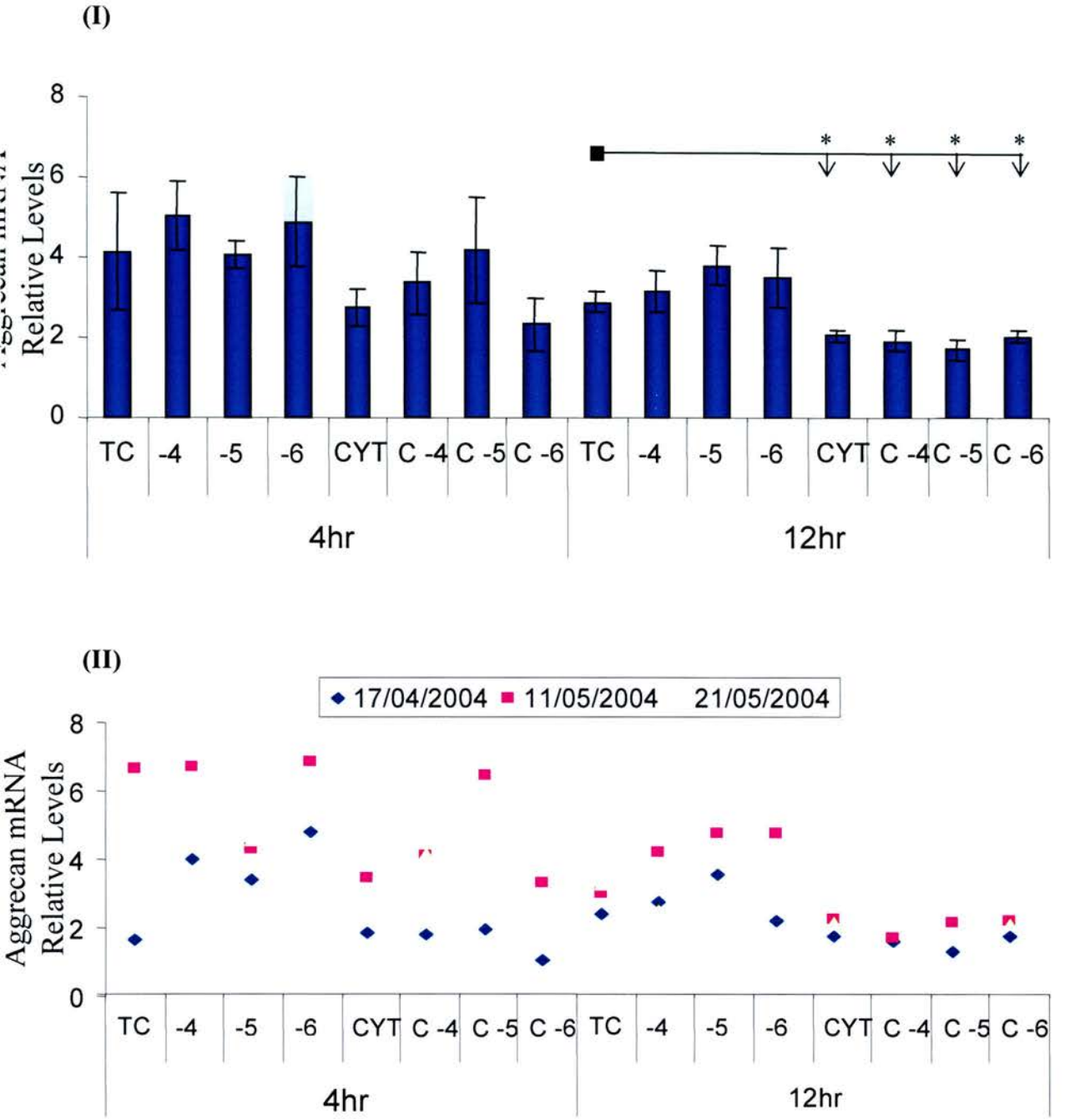


Figure 5.2.13 - Aggrecan mRNA levels detected with primer/probe set 2 following incubation with CYT and the iNOS inhibitor AR-C102222.
(I) = Pooled data±SEM. (II) = Individual experimental values.
TC = Unstimulated control. CYT and C = Cytokine cocktail stimulation. –4 = 10^{-4}M Inhibitor. –5 = 10^{-5}M Inhibitor. –6 = 10^{-6}M Inhibitor. (n=3) *$p<0.05$ (paired t test used).

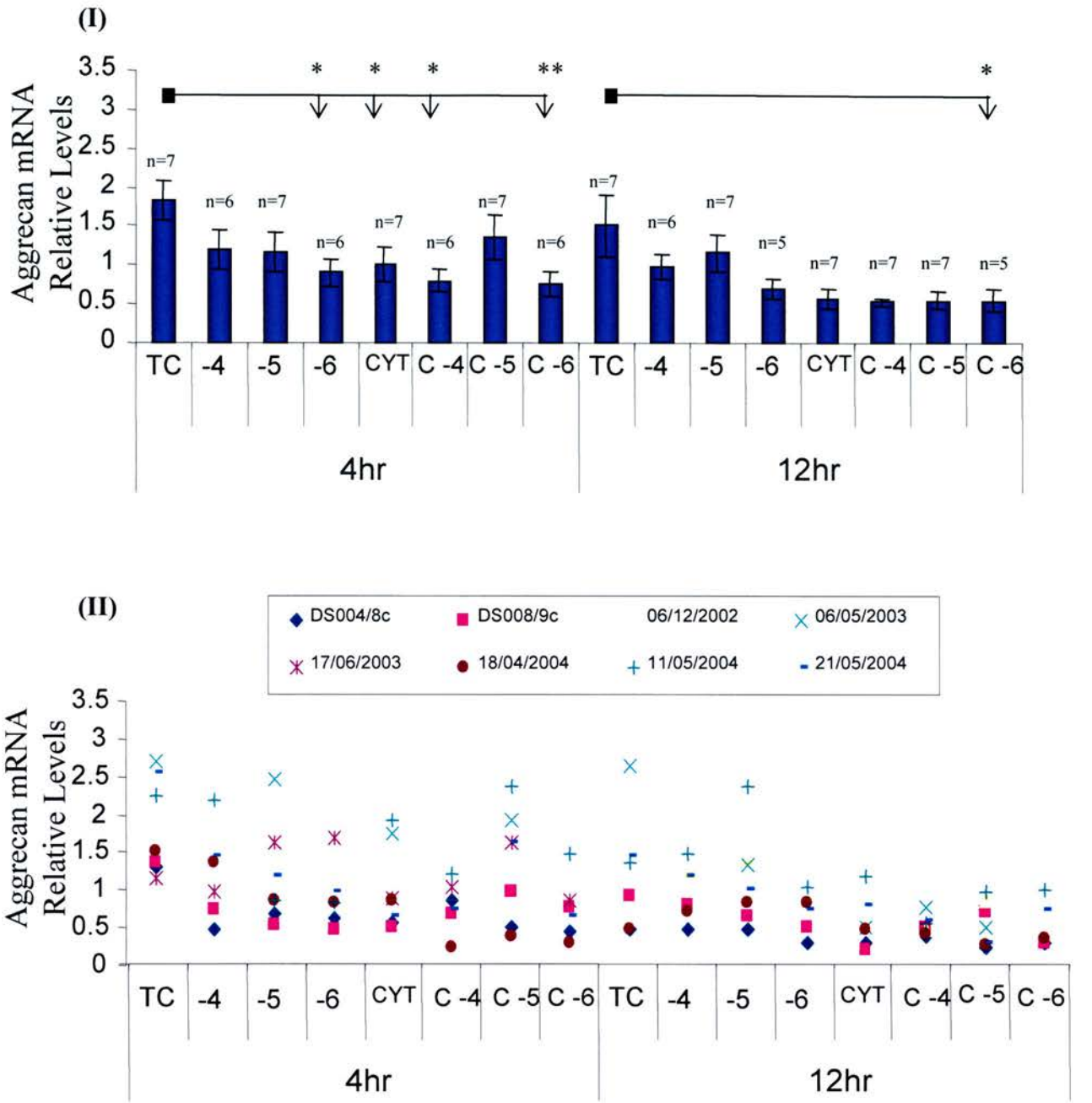


Figure 5.2.14 – Pooled aggrecan mRNA levels detected with primer/probe set 1 following incubation with CYT and the iNOS inhibitor AR-C102222.
(I) = Pooled data±SEM. (II) = Individual experimental values.
TC = Unstimulated control. CYT and C = Cytokine cocktail stimulation. –4 = 10^{-4}M Inhibitor. –5 = 10^{-5}M Inhibitor. –6 = 10^{-6}M Inhibitor. *p<0.05 **p<0.01.

5.2.3b – Effects of the NO donor SNAP on aggrecan mRNA levels

Primary OA HAC were incubated with the NO donor SNAP at 10-1000μM and aggrecan mRNA levels were detected with primer/probe set 1. Nitrite levels in the medium were detected using the Griess assay as a measure of NO released.

The Griess assay showed that incubation with SNAP from 10-1000μM increased the levels of nitrite detected in the medium above unstimulated controls (**Figure 5.2.16**). Nitrite levels of 3.4±0.6, 15.8±6.3 and 63.6±2.9μM were detected following incubation with SNAP concentrations from 10, 100 and 1000μM at 4 hours ($p=0.04$, $p=0.13$ and $p=0.002$ respectively). Nitrite levels of 2.3±0.3, 14.8±1.2 and 104.7±23.1μM were detected following incubation with SNAP concentrations from 10, 100 and 1000μM at 12 hours ($p=0.02$, $p=0.007$ and $p=0.045$ respectively).

Aggecan mRNA levels at 4 hours were not altered from unstimulated control levels following incubation with SNAP at 10, 100 or 1000μM at 4 or 12 hours (**Figure 5.2.15**). One exception to this followed 1000μM SNAP incubation for 12 hours where aggrecan mRNA levels were 2 fold higher than unstimulated control levels ($p=0.03$). Individual experimental values revealed that one sample (DS008/9c) showed a 3.2 fold elevated aggrecan mRNA level compared to the unstimulated control, which remained elevated at 100 and 1000μM SNAP. The other two samples (DS004/8c and 28/6/2003) showed increased aggrecan mRNA levels above control levels only following 1000μM SNAP at 12 hours.

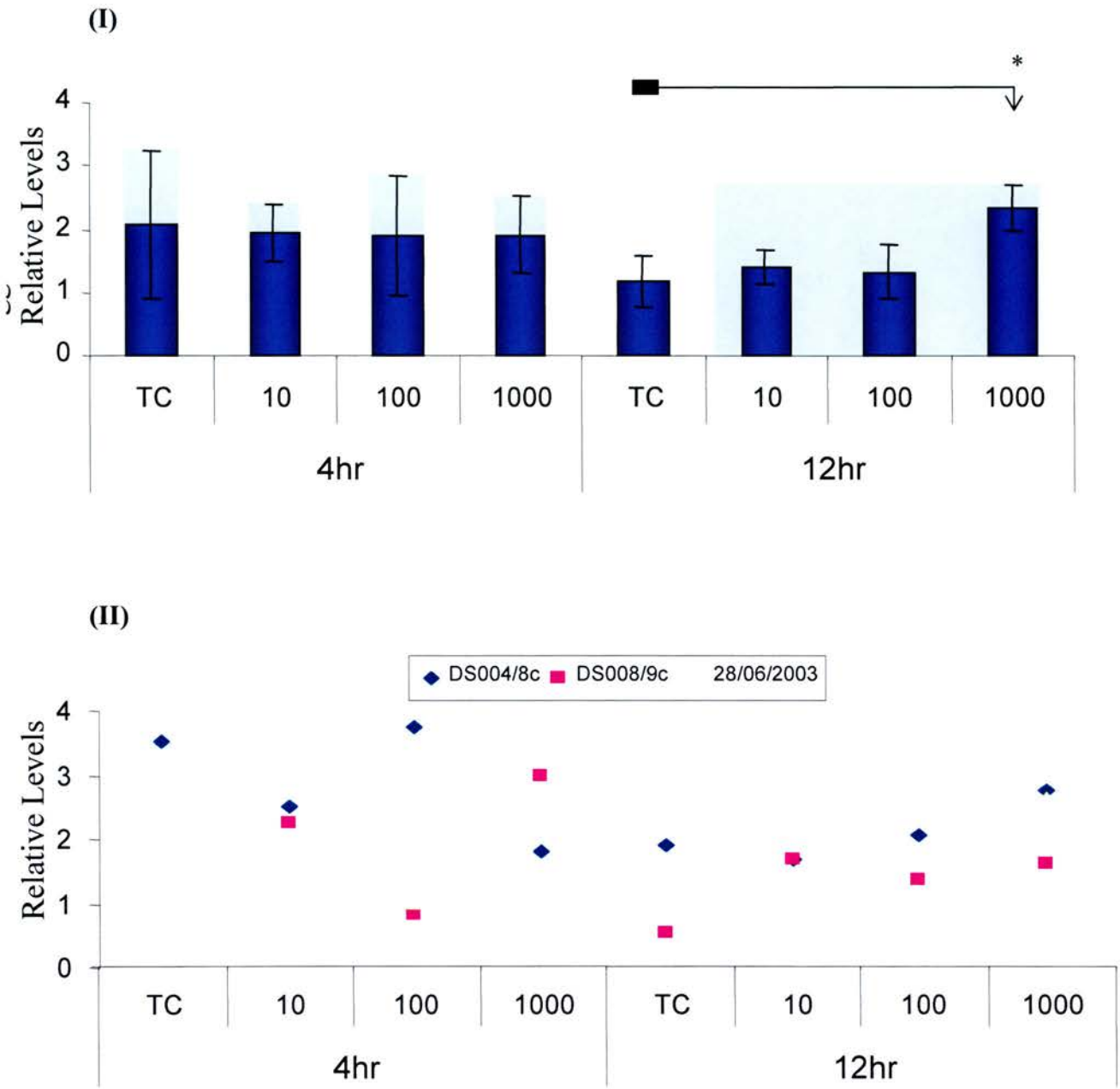


Figure 5.2.15 – Aggrecan mRNA levels detected with primer/probe set 1 following incubation with the NO donor SNAP. TC=Unstimulated control. SNAP values in μM. (n=3). *$p<0.05$ (paired t test used).

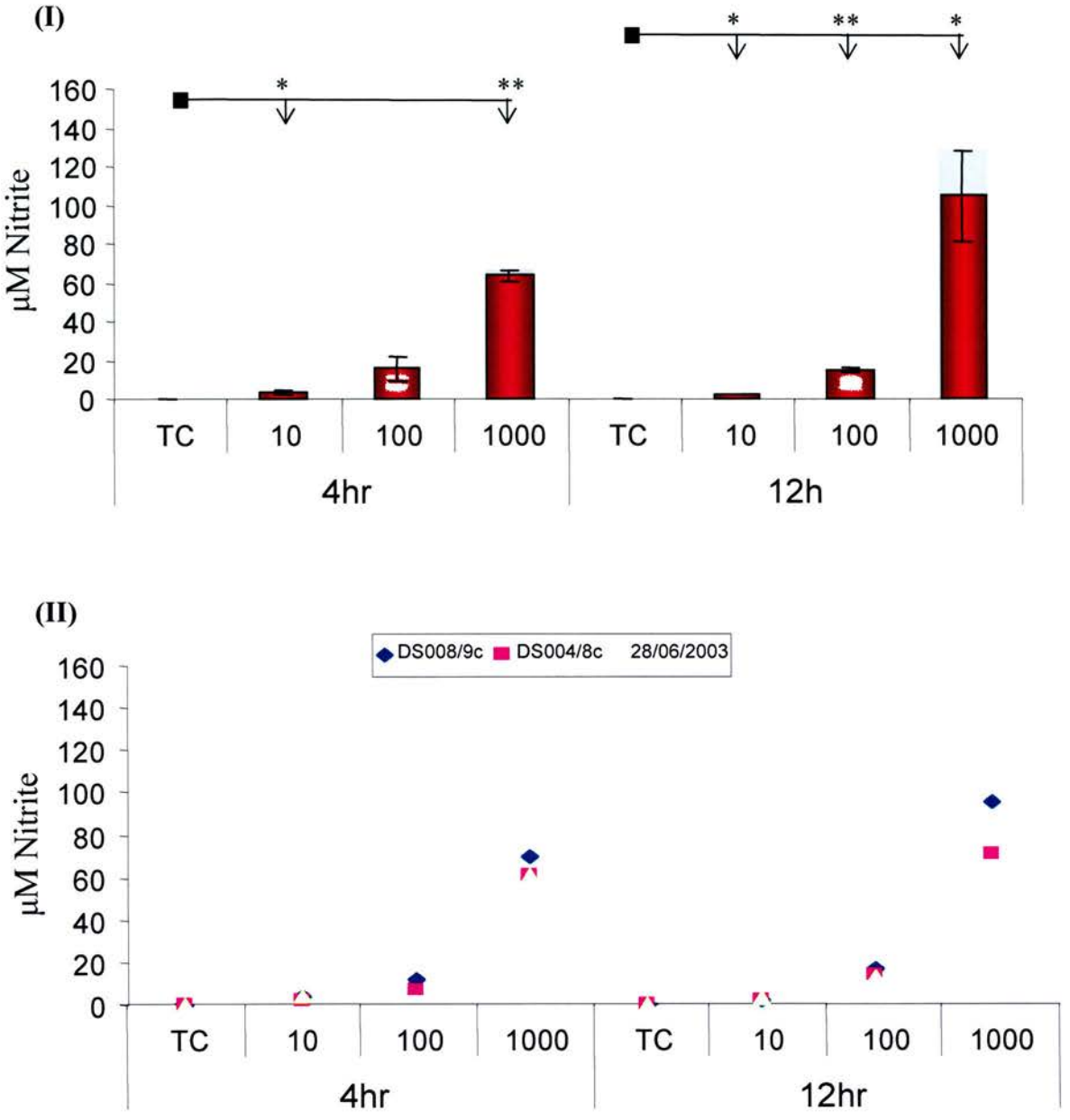


Figure 5.2.16 – Nitrite levels detected by the Griess assay following incubation with the NO donor SNAP. TC=Unstimulated control. SNAP values in μM. (n=3). *p<0.05 **p<0.01 (paired t test used).

5.3 - Discussion of aggrecan mRNA levels following CYT, IL-1β, MS, iNOS inhibitor and an NO donor

There have been a number of studies which have examined the effects of different mechanical loads on proteoglycan (PG) synthesis in chondrocytes and cartilage. The importance of mechanical loading in the maintenance of articular cartilage homeostasis was highlighted in a study by Behrens et al (1989). The complete immobilisation of canine legs decreased the levels of PG synthesis, and a cast that allowed limited motion showed a smaller depression in PG synthesis. The application of cyclic loads can significantly enhance PG synthesis (Sauerland et al 2003), while increased magnitude of load and the application of static compression decreases the level of PG synthesis (Wong et al 1999).

The possible involvement of NO and mechanical strain in the regulation of PG synthesis has been addressed in several studies (Chapter 1). Aggrecan is the major PG in cartilage, so it is likely that any changes in total PG synthesis involve alterations in the production of this molecule. This, however, is not necessarily the case and some studies have therefore investigated the effect mechanical stimulation has on aggrecan mRNA levels (**Table 5.1**). These show that cyclically applied load generally increases aggrecan mRNA levels. Short term static loads initially increase aggrecan mRNA whereas longer term static loading decreases aggrecan mRNA levels.

Taqman real-time PCR analysis of primary OA HAC revealed that mRNA for aggrecan could be detected using two separate primer/probe sets. The two primer/probe sets detect the same molecule within these experiments from the same samples. Although they do generally show similar trends, slight differences are seen between the two that are likely to be due to differences in efficiency of detection. Exposure of these cells to mechanical strain alone, CYT, IL-1β and MS in combination with CYT or IL-1β caused a decrease in the level of aggrecan mRNA when compared to unstimulated controls.

Addition of the β1 integrin function blocking antibody alone was associated with increases in aggrecan mRNA levels of 3.1 and 2.8 fold using primer/probe sets 1 and

2. This suggests that the antibody may have some independent effect on aggrecan mRNA levels. However the result comes from a single experiment. Neither the β1 integrin function blocking antibody or the monoclonal IL-4 neutralising antibody appeared to influence the decreased aggrecan mRNA levels following CYT stimulation.

The sample labelled 22/3/04 (**Figures 5.2.5 and 5.2.6**) and the sample labelled 18/4/04 (**Figures 5.2.8 and 5.2.9**) showed different responses to the application of cytokines and mechanical load than the others under the same conditions. Many factors can influence the cells from a particular sample including the site the cartilage is taken from, as well as the age and gender of the donor. An example of this is the difference in PG synthesis seen following cyclic loading of equine chondrocytes from high and low load bearing areas of the same joint (Wiseman et al 2003).

There are two studies which have looked at aggrecan mRNA levels following tensile strain which are similar to that used in our system (Lahiji et al 2004; Xu et al 2000). Lahiji et al (2004) showed that OA human articular chondrocytes in monolayer subjected to 24 hours cyclic tensile strain had increased aggrecan mRNA levels, a response not seen in our study. Xu et al (2000) used normal rabbit articular chondrocytes in monolayer and showed that the load alone slightly decreased PG synthesis, however no effect on aggrecan mRNA was seen at 4, 24 and 48 hours. This supports our findings where MS did not cause increased aggrecan mRNA levels, actually slightly decreasing them, at 4 and 12 hours. Millward-Sadler et al (2000b) also showed that no alteration in aggrecan mRNA levels were seen due to 0.33Hz MS in OA HAC, although increased aggrecan mRNA was seen in normal HAC. Chowdhury et al (2004) showed increased PG synthesis due to mechanical strain in primary human chondrocytes in agarose constructs, with strain acting through an integrin dependent mechanism. Several studies have shown that strain influences aggrecan mRNA levels in bovine chondrocytes (**Table 5.1**). Lee MS et al (2003a and 2003b) also used OA human articular chondrocytes in monolayer, showing alterations in aggrecan mRNA levels due to both constant shear stress and intermittent hydrostatic pressure.

Several studies, including our own, have shown decreased aggrecan mRNA levels associated with the action of proinflammatory cytokines, which are known to induce NO (Table 5.1). There are several mechanisms by which NO could potentially influence the level of PG synthesis.

Mechanisms by which NO can influence proteoglycan synthesis

The increased PG synthesis due to mechanical stimulation (Table 5.1 and 5.2) could be disrupted by NO through its interaction with mechanical signalling pathway molecules. The prevention of focal adhesion kinase (FAK) recruitment to the focal adhesion complex (FAC) (Clancy et al 1997) and FAC disruption by NO in bovine chondrocytes without influencing integrin expression, distribution or attachment to Fn (Clancy et al 1997) will influence mechanical signalling. The actin cytoskeleton is important in bringing signalling molecules to the FAC and so allowing interactions resulting in signal transduction. Inhibition of actin polymerisation through NO mediated ADP-ribosylation (Clancy et al 1995) prevents the anchorage of these molecules near to the FAC. The application of cyclic stretch has been shown to activate extracellular regulated kinase (ERK) 1/2, c-Jun NH_2 terminal kinase (JNK) and p38 mitogen activated protein kinase (MAPK) (Ingram et al 2000b; Ingram et al 2000c; Ingram et al 2000d) in mesangial cells. NO donors such as SNAP inhibit this activation and prevent the downstream activation of the transcription factors AP-1 and NFκB. MAPK activation requires an intact actin cytoskeleton, and it may be through inhibition of cytoskeletal assembly that NO is having its effect. Zhou et al (our lab - submitted for publication) showed inhibition of the upregulation of PG synthesis following MS by the MAPK inhibitor SB203580 in transformed chondrocyte cell lines. SNAP also decreases protein kinase c (PKC) activity, a response that requires an intact cytoskeleton, the very structure it is shown to disrupt (Gopalakrishna et al 1993; Kim et al 2003; Kim and Chun 2003). Xu et al (2000) suggested that preincubation with cytokine inhibits the decreased iNOS mRNA and nitrite following simultaneous strain and cytokine in rabbit chondrocytes. This may be due to the fact that NO disrupts the mechanical signalling pathways before strain is applied, with the strain being sensed at a quicker rate than cytokine signalling can occur. This suggests that the simultaneous application of strain and CYT in our system allows strain to exert its effects on cytokine signalling before NO can cause disruption.

The regulation of PG synthesis levels may also be explained by the action of NO on insulin-like growth factor (IGF-1) signalling. NO from various sources, including SNAP and IL-1β, decrease PG synthesis associated with the incubation of chondrocytes with IGF-1 (Studer et al 2000). This was shown to be mediated through

the reaction of NO with the sulfhydryl groups on the cysteines of the IGF-1 receptor kinase, forming s-nitrosothiols that may cause functional modification, preventing receptor autophosphorylation and so inhibiting downstream signalling (Studer 2004). Indeed, decreasing the intracellular NO regulator, glutathione, increases the effect of NO on preventing IGF-1 mediated PG synthesis. So by inhibiting the signalling and action of growth factors NO may decrease the level of PG synthesis. The use of iNOS knockout mice have highlighted the importance of NO in decreased PG synthesis due to IGF-1, where the knockouts respond by increased PG synthesis in an almost normal fashion (van den Berg et al 1999).

NO can also have effects on PG levels through promoting its degradation. This can be mediated through influencing the activity of the proinflammatory transcription factor NFκB. This has been shown to occur via the redox derivatives of NO, where peroxynitrite production promotes NFκB activation through sustaining p65 nuclear translocation, whereas S-nitrocysteine inhibits NFκB in bovine chondrocytes stimulated with a proinflammatory cytokine mix (Clancy et al 2004). Agarwal et al (2001) showed that cyclic tensile strain prevented the inhibition of PG synthesis caused by IL-1β in rabbit temparomandibular junction chondrocytes in monolayer culture. A later study (Agarwal et al 2004) applied both low (4-8%) and high (15-18%) cyclic tensile strain to these cells, showing that differential strain regimen can either inhibit (low) or mimic (high) the effects of IL-1β by inhibiting (Low) and activating (high) the transcription factor NFκB. Van't Hof et al (2000) showed iNOS KO mice had defects in bone resorption due to IL-1β, with abnormalities in IL-1β induced NFκB p65 subunit nuclear translocation and DNA binding which were reversed by treatment with SNAP.

Tomita et al (2001) showed rabbit chondrocyte respiration and ATP synthesis were inhibited by NO, particularly in low oxygen conditions similar to those seen in cartilage, with the presence of NO or specific mitochondrial electron transport inhibitors decreasing PG synthesis. Johnson et al (2000) showed NO and peroxynitrite donors decrease respiration and ATP generation in chondrocytes, with mitochondrial oxidative phosphorylation inhibitors decreasing PG synthesis. Stefanovic-Racic et al (1994) showed rabbit articular chondrocytes plus IL-1β produce NO and lactate, with decreased ATP levels. L-NMA, an iNOS inhibitor,

blunts the glycolytic response while SNAP, an NO donor, increased lactate to the same extent as IL-1β. PG synthesis rates are therefore influenced by the action of NO, in part through regulation of intracellular ATP concentration and may be influenced by changes in pericellular pH due to lactate production.

There are therefore several ways in which NO may interfere with PG synthesis in chondrocytes. Mechanical stimulation decreases the production of iNOS mRNA, protein and nitrite following CYT and IL-1β stimulation in our study (Chapter 4). It is possible that mechanical strain may regulate PG synthesis through its control of the production of NO. Indeed, the simultaneous application of mechanical strain and cytokine stimulation has been shown to reverse the decrease in PG synthesis (**Table 1.4**) and aggrecan mRNA (**Table 5.1**) caused by proinflammatory cytokines. It would have been reasonable, therefore, to suggest that through the decrease of NO production, mechanical strain should allow some recovery of the aggrecan mRNA levels towards those seen in unstimulated cells. Our study, however, shows no such recovery in aggrecan mRNA following the simultaneous application of CYT plus MS or IL-1β plus MS.

It is possible that alterations in aggrecan mRNA may actually be missed due to the length of time that strain is applied, as a study by Millward-Sadler et al (2000b) showed that alteration occurred within the first hour following the application of mechanical strain for twenty minutes, and were back to unstimulated levels by 3 hours. It is also possible that the fact that these cells come from OA cartilage means an altered response to mechanical stimulation that does not involve changes in aggrecan mRNA levels. Indeed, no changes were seen in aggrecan mRNA levels in the OA chondrocytes studied by Millward-Sadler et al (2000b). However, the application of intermittent hydrostatic pressure to monolayer OA human chondrocytes in a separate study showed a reversal of lipopolysaccharide (LPS) induced decreased aggrecan mRNA levels after 4 hours (Lee MS et al 2003a) and decreased aggrecan mRNA due to shear stress was reversed by the NO inhibitor L-NMA (Lee MS et al 2002).

The application of cyclic strain, as mentioned, is generally anabolic and causes increases in matrix synthesis. However, if the magnitude of strain or the cyclic

pattern is too great and does not allow recovery of the cell before the next cycle it begins to function as a negative factor. The application of a static strain for a short period of time (30 minutes) increases aggrecan mRNA, while longer times (4 hours) lead to decreases (Ragan et al 1999). It must be considered, therefore, that the application of strain using our system (0.33Hz - 2 seconds ON, 1 second OFF), which appears to be anabolic after 20 minutes (Millward-Sadler et al 2000b) may be acting as a negative factor which is seen at 4 and 12 hours. The slight decrease in aggrecan mRNA due to strain alone supports this, however, the decrease would be expected to be greater and accompanied by elevation in NO levels (Lee MS et al 2002). Reversal of iNOS mRNA, protein and nitrite levels due to our strain regimen (chapter 4) is in conflict with this and suggests the strain is acting in an anabolic fashion.

It must also be remembered that these results are drawn directly from mRNA studies without looking at protein levels. It is possible that increased aggrecan mRNA synthesis occurs, but increased degradation also occurs so showing little alteration of the aggrecan mRNA levels. Although mRNA studies can be indicative of events occurring at the protein level, it is the proteins that are secreted and become part of the ECM so are ultimately the important molecules to monitor. Two of the studies discussed above looked at the level of aggrecan protein bound to the cell surface and found increases due to intermittent hydrostatic pressure in monolayer human OA chondrocytes (Ikenoue et al 2003) and bovine monolayer chondrocytes (Smith et al 1996). However, the decrease in the level of cell associated aggrecan due to cytokine induced NO and potential restoration by the application of mechanical stimulation remains to be studied.

Decreases in synthesis of aggrecan can be difficult to monitor. However the breakdown products associated with aggrecan catabolism can be detected and NO is believed to inhibit this breakdown, possibly via the modulation of aggrecanase activity (Bird et al 2000; Cai et al 2002). IL-1β causes increased MMP production in chondrocytes and NOS inhibitors have been shown to increase MMP activity associated with IL-1β stimulation (Stefanovic-Racic et al 1997). This suggests that NO may not only function as a negative regulatory element in the synthesis of PGs, but may also act to prevent PG breakdown by MMPs.

The fact that NO may act as an inhibitor of PG degradation suggests that mechanical stimulation, through the decrease of NO, may act to reverse the inhibition of PG synthesis caused by CYT, but may also increase the rate of degradation so resulting in the lower aggrecan mRNA levels than detected in unstimulated controls.

The use of the CYT or IL-1β stimulation have been shown to induce iNOS synthesis and NO production, however, there is potential for these molecules to interfere with PG levels in an NO independent fashion (Taskiran et al 1994). The effect of NO on the aggrecan mRNA levels was therefore studied using several strategies including the inhibition of iNOS activity and the application of an NO donor.

Author	Cell Type	Mechanical stimulus	Other Stimuli	Response
Xu (2000)	Rabbit chondrocytes in monolayer	CTS (0.05Hz, 6%)	IL-1β	•IL-1β → ↓aggrecan mRNA by 4hr and sustained for 48hr and ↓PG •CTS → Small ↓PG but had no effect on aggrecan mRNA •CTS plus IL-1β → ↑aggrecan mRNA at 4, 24 and 48hrs and ↑PG synthesis •CTS → ↓iNOS due to IL-1β but no connection to aggrecan and PG shown
Lee MS 2003b	Human OA chondrocytes in monolayer	Shear stress (1.64Pa, 2h) followed by Intermittent hydrostatic pressure (10MPa, 1Hz, 4hr/day for 1,2or4days)	SNP	Shear→ ↑NO and ↓aggrecan mRNA IHP→↓NO due to Shear SNP→↓aggrecan mRNA IHP→partially inhibits this SNP mediated decrease in aggrecan mRNA
Lee MS 2003a	Human OA chondrocytes in monolayer	IHP (10MPa, 1Hz, 4hr)	LPS	LPS → ↓ aggrecan mRNA IHP partially reversed this IHP also reversed iNOS due to LPS but no connection to aggrecan shown
Takenoue 2003	Human normal chondrocytes in monolayer	IHP (1, 5, 10MPa, 1Hz, 4hr in 1 day or in each of 4 days)		IHP → ↑aggrecan mRNA and increased cell associated aggrecan protein
Lee MS 2002	Human OA chondrocytes in monolayer	Constant shear stress (1.64Pa, 2, 6 and 24hrs)	L-NMA	Shear stress → ↑iNOS and NO and ↓aggrecan mRNA L-NMA restored aggrecan mRNA levels
Smith 1996	Bovine monolayer chondrocytes	Intermittent (1Hz) and constant hydrostatic pressure (10MPa, 4hr)		IHP → ↑aggrecan mRNA (31%) IHP and CHP increased PG synthesis and cell associated aggrecan protein Increased aggrecan (2fold) when IHP in 1%FCS instead of serum free
Smith 2000	Bovine monolayer chondrocytes	IHP (10MPa, 1Hz, 2, 4, 8, 12, 24hrs)		IHP→ ↑aggrecan mRNA progressively throughout loading to 3fold. 4hr loading for 4days increased aggrecan mRNA further
Valhmu 1998	Bovine cartilage explants	Static compressive strain (0-0.5MPa, 1-24hrs)	cAMP and IP3 inhibitor	1hr strain (0-0.25MPa)→↑aggrecan mRNA 2-3fold (blocked by cAMP and IP3 inhibitors). 1hr 0.5MPa→smaller ↑aggrecan mRNA 24hr strain → aggrecan mRNA not different to control
Jammi 1994	Monolayer bovine chondrocytes	Static hydrostatic pressure (30MPa)		30MPa → ↓aggrecan mRNA and PG synthesis
Koyoda 2003	Bovine chondrocytes in agarose	Static hydrostatic pressure (5MPa, 4hrs)		Strain → ↑aggecan mRNA
Sagan 1999	Bovine cartilage explants	Static mechanical compression (various load magnitude and times)		Increased aggrecan mRNA in initial 30mins, significant decrease from 4-24hrs. Decrease in aggrecan mRNA and PG synthesis with increasing compression magnitude.
Sahiji 2004	Human monolayer chondrocytes	CTS (flexible membrane, 0.5Hz, 7%elongation, 24hrs)		CTS →Increased aggrecan mRNA
Millward-Sadler 2000b	Human normal and OA chondrocytes	CTS (0.33Hz, 3000μstrain)		CTS increases aggrecan mRNA in normal and decreases in OA chondrocytes

Table 5.1 – Studies looking at aggrecan mRNA and protein levels following mechanical stimulation. CTS=Cyclic tensile strain. IHP=Intermittent hydrostatic pressure.

iNOS inhibitor ARC-102222

The use of iNOS inhibitors has allowed the study of the role of NO in both the synthesis and degradation of PGs. Hauselmann et al (1994) used the NOS inhibitor L-NMA, and the NO donor SNAP, to show that NO was partially responsible for IL-1β mediated decreases in PG synthesis in human articular chondrocytes in alginate culture. Lee DA et al (1998) used NOS inhibitors (L-NAME and Dexamethasone) to show that NO partially inhibited PG synthesis in bovine chondrocytes seeded in agarose. The same group (Chowdhury et al 2001; Chowdhury et al 2003) has since shown that the iNOS inhibitor L-NIO inhibited increased PG synthesis following cyclic compressive loading. Liu et al (2001) used L-NMA to show that NO inhibited increases in PG in response to low hydrostatic pressure and was involved in decreased PG due to high hydrostatic pressure in human lumbar disc cubes. Gassner et al (2000), using cyclic tensile strain similar to that used in our system, showed that L-NMA partially restored the inhibition of PG synthesis associated with IL-1β in monolayer rabbit chondrocytes. The only study to show the role of NO in the control of aggrecan (Lee MS et al 2002) used L-NMA to show shear stress induced NO was responsible for decreased aggrecan mRNA levels. An *in vivo* study (Presle et al 1999) showed that IL-1β injections into rat knee caused increased NO leading to decreased PG synthesis. The iNOS inhibitors L-NMA and L-NIO decreased NO production and partially restored PG synthesis.

Stefanovic-Racic et al (1997) showed that in rabbit cartilage shavings IL-1β led to decreased PG synthesis and increased degradation. NOS inhibition with L-NMA and thiocitrulline reversed the effects on PG synthesis, but increased degradation by collagenase, stromelysin and gelatinase (MMP) activation. The same group found similar results in bovine cartilage slices (Stefanocvic-Racic et al 1996) and primary human chondrocytes (Hauselmann et al 1998). This suggests that NO can also have protective effects through prevention of cartilage degradation.

In our study with the iNOS inhibitor (ARC-102222) where data from primer/probe set 1 was pooled (Figure 5.2.14) showed that the inhibitor itself appeared to slightly reduce aggrecan mRNA levels compared to the unstimulated control at both 4 and 12 hours. The slight decrease due to inhibitor alone suggests that it is interfering with

mRNA synthesis in some way. This is supported by the fact that the highest concentration of the inhibitor, 10^{-4}M, also lowered iNOS mRNA levels (chapter 4). It may therefore be acting by an NO independent, non-specific inhibition of gene transcription. However, GAPDH levels remain unaffected, suggesting that there may be a specific mechanism through which the inhibitor could be acting, such as through the inhibition of action of another enzyme involved in mechanical or cytokine signalling. It is possible that, despite the lack of detection of NO without cytokine stimulation, some basal production occurs that may be blocked by the inhibitor. This would, however, be expected to cause a slight increase in the levels detected rather than the decrease seen, suggesting some other mechanism of action is more likely.

Significant reduction in aggrecan mRNA levels follows the addition of CYT, and the simultaneous addition of CYT and inhibitor have no significant effect on these levels. The inhibitor was shown in Chapter 4 to decrease iNOS nitrite levels, and as discussed above, NO is involved in the decrease in proteoglycan and aggrecan mRNA due to LPS and IL-1β stimulation. This would suggest that the inhibitor, through its inhibition of NO production, can prevent the action of the cytokine mix in decreasing aggrecan mRNA levels. These alterations in aggrecan mRNA levels are, however, not seen which suggests that the cytokine mix is acting independently of NO to decrease aggrecan mRNA levels. It is also possible that the decreased NO production in these experiments could have a dual effect on aggrecan mRNA levels. The decrease in NO would restore aggrecan mRNA synthesis to unstimulated controls, but it would also interfere with the inhibitory effect of NO on cytokine mediated proteoglycan degradation that may lower aggrecan mRNA levels.

In future experiments levels of aggrecan mRNA following IL-1β stimulation alone in the presence of the inhibitor should be undertaken. It is possible that in these circumstances a reversal of the decrease in aggrecan mRNA could be demonstrated.

NO donor S-Nitroso-N-acetylpenicillamine (SNAP)

Although the iNOS inhibitor did inhibit NO production (Chapter 4) it did not prevent the effects of the cytokines on PG levels. Thus the effect of NO could be masked by an NO independent decrease in aggrecan mRNA. In order to determine the effects of NO alone an NO donor was used.

Several studies have looked at the effect of NO donors on PG synthesis. Hauselmann et al (1994) showed that SNAP mimicked the effects of IL-1β, causing decreased PG synthesis in human articular chondrocytes cultured in alginate. Lee MS et al 2003b) showed that the NO donor sodium nitroprusside (SNP) decreased aggrecan mRNA by 30-40%. Taskiran et al (1994) showed that SNAP mimicked the effect of IL-1β, decreasing PG synthesis in slices of rabbit cartilage. Liu et al (2001) showed that SNAP increased NO and decreased PG synthesis. Two studies by Studer et al (Studer et al 2000; Studer et al 2004) looked at the effects of NO on IGF-1 mediated increases in PG synthesis and showed that the NO donors SNAP, NOC-7 and DETA-NONOate inhibit PG synthesis through prevention of IGF receptor kinase auto and substrate phosphorylation. The iNOS inhibitor L-NMA restored the decrease in PG induced by IL-1β. The NO donor glycerol trinitrate (nitroglycerin) also implicates NO in PG depletion, causing reduced PG within the articular cartilage of sheep *in vivo* (Cake et al 2003).

There are a number of NO donors that have been produced and are commercially available. These each have different properties that allow the release of NO at different rates. SNAP is an NO donor that spontaneously releases NO upon solubilisation in aqueous buffers with a half-life of 10 hours (SIGMA data sheet). This makes it ideal for use in our system, where the effects of NO at 4 and 12 hours are being studied. However, the biological half-life is decreased due to a rise in pH or the presence of metal ions, particularly copper and iron, increasing the decomposition rate (Askew et al 1995). Within our system SNAP will be exposed to metal ions through the opening of ion channels in response to mechanical strain, however these are potassium, calcium and sodium, not copper and iron (Millward-Sadler et al 2000a). The production of NO may also lead to the altered production of energy by the cell, causing lactate production that will alter the pH (Stefanovic-Racic et al

1994). Indeed SNAP increased lactate production to the same extent as IL-1β, and so could potentially induce an increase in its own generation of NO. Therefore it is possible that in our system SNAP could generate NO more quickly than might be initially expected.

SNAP addition led to large increases in the level of nitrite detected in the medium, suggesting that NO release does occur. This production was seen at 4 hours and not greatly increased at 12 hours at the lower concentrations, suggesting early release of NO compared to that seen following CYT stimulation, where significant levels are detected only at 12 hours. This may be important as any change due to NO may occur at a much earlier stage than that following CYT, and so may be missed at the time points measured. Indeed, this may be the case as the level of aggrecan mRNA does not alter following SNAP addition at 4 or 12 hours, with the exception of a slight increase at the highest concentration (1000μM). The level of nitrite detected at this concentration of SNAP is far in excess of that seen following CYT, and would be expected to cause reduction in aggrecan mRNA levels. The reason for this small elevation in aggrecan mRNA levels at 12 hours is unclear, with no response seen at 4 hours. It may be important to note that only small increases were seen in 2 of the samples, whereas one sample (28/6/03) showed a greater increase. This sample showed higher levels of NO release due to SNAP at the highest concentration, which might affect aggrecan mRNA. Potential interaction of NO with other molecules within the cell can not be ruled out. Chenevier-Gobeaux et al (2004) showed that SNAP causes increased hyaluronic acid at 12 hours with little response seen at 6 hours in synovial cells from rheumatoid arthritis patients. The higher concentrations (0.1 and 1mM) induced a greater increase than 0.01mM.
It must also be considered, however, that NO may actually have no effect on the level of aggrecan mRNA within our system.

5.4 - Conclusion

Decreased CYT and IL-1β stimulated iNOS mRNA, protein and nitrite have been demonstrated following MS in these studies (Chapter 4). The addition of CYT and IL-1β decreased the level of aggrecan mRNA detected. The studies have employed mechanical stimulation, an iNOS inhibitor and an NO donor to determine the role of NO on aggrecan mRNA levels. The use of iNOS inhibitors and NO donors by others (Bird et al 2002; Chowdhury et al 2001; Chowdhury et al 2003; Gassner et al 2000; Lee DA et al 1998; Liu et al 2001) has demonstrated the involvement of NO in the decreased PG synthesis due to proinflammatory molecule stimulation. Only one other group has however shown the same response in aggrecan mRNA using SNP (Lee MS et al 2003b) and L-NMA (Lee MS et al 2002) in primary OA human articular chondrocytes cultured in monolayer (**Table 5.1**). Given the evidence suggesting a role for NO in decreasing PG levels it would have been expected that, through the decrease of NO production, MS should allow the aggrecan mRNA levels to return to unstimulated levels. This, however, was not the case and no return towards unstimulated levels was seen following the simultaneous addition of CYT and MS or IL-1β and MS. The possibility for NO independent effects of the cytokines and MS led to further studies to determine the effects of NO on aggrecan mRNA levels. The use of a novel iNOS inhibitor showed efficacy in decreasing the level of NO produced (Chapter 4). However little effect was seen on the decreased aggrecan mRNA levels following CYT stimulation. The NO donor SNAP was used to determine the effects of NO on aggrecan mRNA levels alone, but no decrease was seen. The possible reasons for the lack of response to altered NO levels have been discussed.

Despite all the evidence suggesting a role for NO in PG degradation, the possibility that NO may not be involved in degradation in our system must be considered.

NO-Independent Mechanisms for Decreasing Proteoglycan Synthesis

Inflammatory cytokine stimulation may reduce aggrecan mRNA levels through decreased mRNA transcription, by increased mRNA degradation or by alteration in mRNA stability.

Separation of deep and superficial chondrocytes has suggested that deep cell proteoglycan synthesis is influenced by an NO mechanism alone, whilst superficial cells show IL-1β suppressed proteoglycan synthesis in both an NO-dependent and independent fashion (Chowdhury et al 2003; Hauselmann et al 1998). A similar situation is seen in the inhibition of proteoglycan by IL-1β where NOS inhibitors partially reverse IL-1β induced decreases in proteoglycan synthesis (Taskiran et al 1994) and NO donors partially decrease PG synthesis but not to the levels seen for IL-1β (Oh et al 1998). These studies suggest an NO-independent action. However no mechanism for this has been elucidated. Partial reversal may also occur through the incomplete inhibition of NO production, allowing NO to have residual effects.

It is possible that the environment within the chondrocytes steers the activity of NO in a particular direction. Molecules such as glutathione, which mops up NO, could bind it and prevent its effects within the cell. If this were indeed the case detectable NO levels would be reduced as less would accumulate in the medium, as seen at 4 hours in our experiments. However, detectable levels at 12 hours suggest that any effects would simply delay NO reduction of PG. The simultaneous production of the superoxide radical (O_2^-) and NO has been shown to be required for IL-1β to inhibit PG synthesis, suggesting a role for peroxynitrite (Oh et al 1998). It may be that O_2^- is not produced in our system, so NO does not affect proteoglycan synthesis. The presence of superoxide dismutase (SOD) may be important, as this enzyme scavenges oxygen free radicals and so may influence the levels of O_2^- available for reaction with NO (Zhang and Rosenberg 2002).

It is also important to consider the effects of mechanical strain on cells independent of its action on NO. Changes in cytoskeletal organisation, cell shape and nuclear diameter were seen due to compressive strain of bovine chondrocytes in agarose constructs (Lee DA et al 2000). High hydrostatic pressure disrupts the cytoskeletal

network (Parkkinen et al 1995) and Golgi complex (Parkkinen et al 1993). Constant high hydrostatic pressure decreases aggrecan mRNA and proteoglycan synthesis (Lammi et al 1994) and effects on cell shape, intracellular transport and protein modification may account for some of its inhibitory effect on proteoglycan synthesis. Indeed, excessive mechanical load effects the MMP/TIMP ratio and the expression of IL-1β and TNFα, and so influences cartilage metabolism (Honda et al 2000).

Summary of findings

- Aggrecan mRNA levels are reduced by both CYT and IL-1β stimulation.

- The lower levels of CYT induced NO seen following mechanical stimulation or incubation with the iNOS inhibitor did not restore CYT mediated decreases in aggrecan mRNA levels.

- The NO donor SNAP did not alter aggrecan mRNA levels.

Chapter 6 – Conclusions and general discussion

(I) The transformed chondrocyte cell lines C20A4 and C28I2 show detectable levels of e, n and iNOS mRNA, but do not produce NOS isoform protein or nitrite.

(II) Primary osteoarthritic human articular chondrocytes produce e, n and iNOS mRNA, however no e or nNOS protein is detected.

(III) Primary osteoarthritic human articular chondrocytes produce iNOS mRNA, protein and nitrite following stimulation with a proinflammatory cytokine cocktail or IL-1β alone.

(IV) The application of mechanical stimulation (0.33Hz, 30,000μstrain) reduces the level of iNOS seen following CYT or IL-1β stimulation. This decrease occurs through a β1 integrin and IL-4 independent mechanism.

(V) A novel iNOS inhibitor, AR-C102222, inhibits iNOS enzyme activity without affecting its mRNA or protein expression.

(VI) Aggrecan mRNA levels are reduced by both CYT and IL-1β stimulation.

(VII) The lower levels of CYT induced NO seen following mechanical stimulation or incubation with the iNOS inhibitor did not restore CYT mediated decreases in aggrecan mRNA levels.

(VIII) The NO donor SNAP did not alter aggrecan mRNA levels.

The chondrocyte can sense and respond to mechanical signals through its attachment to the extracellular matrix (ECM) causing the altered synthesis and turnover of ECM molecules. The importance of this interaction is seen as the loss of ECM contact results in cell death. The extracellular environment and the mechanical stimuli the cells are exposed to are altered in osteoarthritis (OA), possibly contributing to the degeneration of articular cartilage. Understanding the response of cells from OA tissue to mechanical signals may point to novel targets for the development therapeutic strategies for the treatment of OA.

The avascular, aneural and alymphatic structure of articular cartilage requires that molecules enter the tissue by diffusion, a process which is enhanced during the movement of water which accompanies the application of load to the tissue. As chondrocytes are the only cell type in cartilage and are sparsely distributed they must communicate through the release of soluble mediators. The detection of molecules such as growth factors and cytokines by the chondrocytes alters their gene expression patterns and so influences the regulation of cartilage homeostasis. The concentrations of proinflammatory molecules are enhanced in OA, causing an imbalance between matrix synthesis and degradation that disrupts cartilage homeostasis and results in cartilage breakdown and OA progression. The inhibition of the action of these proinflammatory molecules has been highlighted as a mechanism through which the progression of OA could be prevented.

This study has investigated the interaction between two important regulators of chondrocyte function; mechanical signalling and cytokine signalling.

The transformed human chondrocyte cell lines C20A4 and C28I2 did not produce detectable levels of endothelial (e), neuronal (n) or inducible (i) nitric oxide synthase (NOS) protein. NOS isoform protein and enzyme activity was not detected following 4 or 12 hour stimulation with a proinflammatory cytokine cocktail (CYT) or mechanical stimulation (0.33Hz, 30,000μstrain) (MS). NOS isoform mRNA was detected, however this was carried out on a single occasion preventing any analysis of variation in the levels detected. The lack of detectable NOS protein or enzyme activity, however, suggests that there are alterations in the signal transduction pathways or alterations in protein production at some stage from mRNA stabilisation

to protein synthesis and degradation. The fact that these cells are virally transformed and de-differentiation in some respects following their long term culture in monolayer are possible reasons for their lack of response.

Primary osteoarthritic human articular chondrocytes (HAC) did not produce NOS isoform protein or show enzyme activity when unstimulated despite the proinflammatory rich *in vivo* environment from which they were isolated. CYT stimulation and IL-1β alone significantly increased iNOS mRNA, protein and nitrite levels. The application of simultaneous CYT and MS resulted in levels of iNOS mRNA, protein and nitrite that were elevated above unstimulated controls, but were significantly lower than following CYT alone. While previous studies have shown a reversal of single cytokine induced NO (both IL-1β and TNFα), this is the first study to show mechanical stimulation reverses iNOS levels induced by a proinflammatory cytokine mix of IL-1β, TNFα, IL-6 and IFNγ. The level of iNOS mRNA, protein and nitrite is similar when using the cytokine mix or IL-1β alone which suggests the IL-1β may be the main inducer of iNOS in this system. The stimulation of iNOS in chondrocytes has been shown using IL-1β and TNFα alone while the other cytokines in the mix, IFNγ and IL-6, do not stimulate iNOS alone but enhance its induction by IL-1β and TNFα. It may therefore be that maximal iNOS stimulation can be achieved by CYT or by IL-1β alone. The reversal of CYT induced iNOS levels suggests that the application of MS may be interfering with the CYT induced signal transduction pathways that lead to elevated iNOS. This could be mediated through either an iNOS specific action or a more general interference in cytokine signalling. It would be of interest to look individually at each of the cytokines and their effects on iNOS induction as well as the effect of mechanical strain in each case. This would allow more detailed identification of the effects of each of the cytokines as well as those influenced by MS.

MS has been shown to induce membrane depolarisation in OA chondrocytes through a mechanism that involves the autocrine/paracrine production of the cytokine IL-4, IL-1β and α5β1 integrin (Millward-Sadler et al 2000a). The close association that has been described between the integrin-mediated mechanical signalling molecules and cytokine receptors provides a possible mechanism for signalling interactions (Lo et al 1998). An IL-4 neutralising antibody and a β1 integrin function blocking antibody

were used to investigate possible mechanisms by which MS was interfering with CYT-induced iNOS production. These studies demonstrated that MS is acting through a β1 integrin and IL-4 independent pathway to decrease CYT induced iNOS. The question which then has to be addressed is 'How are the mechanical signals interfering with CYT induced iNOS?'. There are other receptors on the surface of the chondrocyte that may be involved in the sensing of these mechanical signals, including other integrins (Loeser et al 1995) and CD44 (Knudson and Loeser 2002). The activation of transmembrane ion channels following mechanical stimulation has been described (Wright et al 1996). It is possible that these channels could cause a widespread alteration in the activation of signalling molecules within the cell. It is also possible that through the control of intracellular pH these channels could alter the activity of the NOS enzyme (Conte 2003). This could account for the decreased nitrite detected in our studies, but not the decreased iNOS mRNA and protein levels. The phosphorylation and activation of signalling molecules, particularly the mitogen activated protein kinases (MAPK), protein kinase C (PKC) and integrin-linked kinase (ILK), are involved in both CYT and mechanical signalling pathways. It is possible that, through alterations in the activation pattern of these molecules, MS could interfere with CYT induced iNOS. Another molecule that is important in CYT induced iNOS production is the transcription factor NFκB. This has been shown to be influenced by mechanical strain (Agarwal et al 2004) and may represent a mechanism by which MS can interfere with CYT induced iNOS. Indeed, two of the proinflammatory cytokines that can induce iNOS, IL-1β and TNFα, can also stimulate NFκB activation, and NFκB is an important molecule in the induction of iNOS mRNA synthesis. It may however be that these pathways do not interact, but rather that they both produce molecules that bind the iNOS promoter, which has multiple control elements, and that the combined effects of these result in the lower level of iNOS production following simultaneous CYT and MS than following CYT alone. The inhibition of cell surface molecules that sense mechanical signals, or any of the molecules potentially involved in intracellular signalling would allow identification of the mechanism through which mechanical strain is acting to decrease CYT-induced iNOS levels. Stimulation with single cytokines would allow a better determination of this effect.

As previously mentioned, chondrocytes are responsible for the maintenance of cartilage matrix homeostasis. This involves the synthesis and degradation of molecules important to the structure of cartilage including the proteoglycans (PG). Proinflammatory cytokines have been implicated in the alteration of proteoglycan synthesis, with the production of NO suggested by several groups as a mechanism through which this may occur. The production of NO has been implicated in the progression of osteoarthritis, with detectable levels found in human OA and inhibitors shown to have beneficial effects in animal models. The effects of NO, however, have been shown to be complicated. In addition to being able to decrease the synthesis of proteoglycans, NO also inhibits the activation of matrix degrading enzymes. Mechanical loads of a physiological magnitude and frequency increase PG synthesis. However, static loads and excessive magnitudes or frequencies can lead to decreased proteoglycan synthesis.

Both cytokine and mechanical signals alter matrix homeostasis by influencing proteoglycan synthesis and degradation. The production of iNOS due to CYT stimulation and the interference by MS in our system led to the investigation of the possible role of NO on the major proteoglycan in cartilage, aggrecan. Aggrecan mRNA levels were studied using mechanical stimulation, an iNOS inhibitor and an NO donor. Several studies have shown that cyclical strain reverses both cytokine induced NO levels and the decreased aggrecan mRNA levels associated with IL-1β and LPS. However, a link between these has only been shown by one group previously. They initially used the iNOS inhibitor L-NMA to show reversal of shear stress-induced decreased aggrecan mRNA levels, and then demonstrated that the NO donor sodium nitroprusside decreased aggrecan mRNA levels (Lee MS et al 2002, Lee MS et al 2003b).

Following CYT stimulation aggrecan mRNA levels were decreased in our study. The evidence that suggests NO inhibits PG synthesis, along with the fact that mechanical stimulation decreases the NO levels associated with CYT, led to the theory that mechanical stimulation may reverse the decreased aggrecan mRNA levels following CYT stimulation. This was not seen, however, suggesting that the CYT may be acting in an NO-independent fashion to decrease PG synthesis. It may be that the individual cytokines in the cytokine mix are having several independent effects on

aggrecan mRNA levels. Mechanical stimulation did show some reversal of the IL-1β mediated decrease in aggrecan mRNA levels, but this was seen using only one of the two primer/probe sets and did not reach statistical significance. This suggests that it may be important to investigate the effects of each of the cytokines individually. The potential complications of using a the cytokine mix are highlighted in previous studies which demonstrated that IL-6 (Legendre 278, 2903 2003), IFNγ and TNFα (Dodge 41, 274 1998), as well as IL-1β (Xu 2000) all decreased aggrecan mRNA levels, with NO only implicated in the IL-1β mediated decrease. It is possible, therefore, that the decreased NO due to mechanical strain does partially reverse the IL-1β mediated decrease in aggrecan mRNA levels but the other cytokines act to keep the levels below that seen in unstimulated cells. Investigation of the effects of each of the cytokines individually and the influence of MS would allow a better understanding of the role each plays in regulating aggrecan mRNA levels. It is also important to consider that mechanical stimulation itself can result in structural changes within the cell, disrupting the cytoskeleton and golgi and so influence proteoglycan synthesis. The experiments reported in this thesis only measure the level of aggrecan mRNA, and not the rate of synthesis or degradation. It has been shown that NO may act as an inhibitor of PG degradation as well as causing decreased PG synthesis. By decreasing NO levels, mechanical strain may act to reverse the inhibition of PG synthesis caused by CYT, but may also increase the rate of degradation resulting in aggrecan mRNA levels remaining lower than unstimulated controls.

The iNOS inhibitor, AR-C102222, was initially shown to have little effect on iNOS mRNA or protein levels but showed a dose dependent decrease in iNOS enzyme activity associated with CYT stimulation. The use of iNOS inhibitors has previously been reported to reverse the decreased proteoglycan synthesis associated with IL-1β stimulation (Gassner et al 2000). The decrease in NO levels due to AR-C102222, however, did not affect the aggrecan mRNA levels detected. The use of the cytokine cocktail allows for the activation of multiple signalling pathways that may allow alterations in aggrecan mRNA levels through NO-independent mechanisms. The study of the effects of the inhibitor on the single cytokine mediated decreased proteoglycan levels would address this problem.

The NO donor SNAP was used to identify the effect of NO alone on aggrecan mRNA levels. SNAP has previously been shown to mimic the effects of IL-1β, decreasing PG synthesis (Hauselmann et al 1994). There was no alteration in aggrecan mRNA levels following SNAP incubation at a range of concentrations. The experimental conditions may however have resulted in an increased rate of NO release from SNAP inducing alterations in aggrecan mRNA levels that were restored before they were measured at 4 and 12 hours. Alteration in the culture conditions or the use of a range of NO donors at a series of time-points would allow a better evaluation of the effects of NO on aggrecan mRNA levels.

The iNOS inhibitor and SNAP experiments suggest that the cytokines may be acting in an NO-independent fashion to decrease aggrecan mRNA levels. This has been previously suggested by other studies that have shown NOS inhibition only partially reverses IL-1β mediated decreases in PG synthesis. Direct effects on gene promoter activity through transcription factor induction is likely to be one mechanism through which these proinflammatory cytokines could influence PG synthesis. The aggrecan promoter contains two NFκB sites as well as STAT and AP-1 sites (Valhmu 273, 6196 1998). These transcription factors are induced by the proinflammatory cytokines, providing a mechanism through which they could downregulate aggrecan gene expression. Four shear stress response elements have also been identified that provide a mechanism by which MS can influence aggrecan mRNA levels (Valhmu 1998).

It has also been suggested that the superoxide radical is required for NO mediated decrease in PG synthesis. Reduced levels of superoxide would therefore prevent NO from having a negative effect. This could result from the action of the enzyme superoxide dismutase (SOD) that scavenges free oxygen radicals. Detection of SOD within chondrocytes may therefore explain the lack of effect of SNAP. Mechanical strain decreases cytokine induced NO levels. However, decreases in SOD levels or activity due to mechanical strain may increase the level of proteoglycan degradation. The level of glutathione within the cell may also be important and should be monitored as it binds NO and inhibits its action within the cell.

Although the C20A4 and C28I2 cell lines can be induced to produce iNOS mRNA by proinflammatory cytokines, they do not produce iNOS protein or nitrite under any conditions tested. These cells have also been shown to produce PG synthesis in response to mechanical stimulation (Zhou et al submitted for publication). This suggests that they may be used as a model to investigate the effects of cytokine stimulation on PG synthesis in the absence of iNOS.

The effects of NO within cartilage are difficult to understand as both pro-inflammatory and anti-inflammatory effects have been reported in different systems. Indeed, even within the same system NO can act to decrease proteoglycan synthesis while preventing proteoglycan breakdown through inhibition of MMP activity. NO is a highly reactive molecule that can have a variety of effects depending upon the molecules present within its environment. The ability of NO to react with cysteine and iron containing molecules provides a mechanism through which it can influence many molecules within the cell. Understanding NO and the possible mechanisms by which it may be functioning in tissues in patients with OA and inflammatory arthritis is therefore an important area for study.

This study has demonstrated that mechanical stimulation can be used as an anti-inflammatory tool that operates through the reversal of cytokine stimulated NO production. However the complex interactions of NO require elucidation in order to determine if the decrease in NO production does indeed have an anti-inflammatory effect. Further study is therefore required to determine the mechanism(s) through which MS inhibits iNOS production.

Chapter 7 - Future work and future directions

The research contained within this thesis provides a number of answers to questions which have been posed throughout the course of the study. The answers to these questions have invariably led to the posing of further questions, and it is these and the potential strategies that could be adopted to address them that this chapter aims to discuss.

The first thing that requires attention is the number of replicates. Human chondrocytes show a large amount of variation in their responses and this influences the level of statistical significance. Thus, increased replicates would increase the power of the results.

The two main areas of this study that would be of interest to further research are;

- How do cytokine and mechanical stimulation pathways interact during iNOS production?

- What are the effects of NO on matrix regulation?

Investigation of the interaction between cytokine and mechanical stimulation

These studies have been carried out in monolayer culture, enabling the extraction of mRNA, protein and assay of enzyme activity immediately at the end of each experiment. This has allowed the investigation of the pathways through which mechanical strain may be influencing the level of iNOS production following cytokine stimulation. We have shown that blocking of the β1 integrin and the release of IL-4 has no effect on this mechanically induced suppression of cytokine induced iNOS. Further studies would need to increase the number of replicates of these experiments as well as investigating other strategies that could be adopted to confirm these results. This could include the use of gene mutational studies to determine the

importance of these molecules or the use of other pharmacological inhibitors. The next step would be to investigate the other mechanisms that may be involved. This would include the use of pharmacological stretch activated ion channel blockers such as apamin, other integrin blocking antibodies as well as blockers of other cell surface molecules such as CD44.
The inhibition of signalling molecules that are common to both pathways using pharmacological inhibitors would also be of interest. The use of knockouts for certain signalling molecules could be beneficial in studying this. The activation of MAPK that lead to NFkB activation is likely to play a role in the interaction between mechanical and cytokine signalling pathways. The uses of mobility shift assays to track the activation of NFkB as well as the production of iNOS could be used as a measure of interaction between mechanical and cytokine signalling.

Gene chip technology where a large number of genes can be simultaneously analysed may be beneficial within these studies, allowing a more global understanding of the interactions between cytokine and mechanical stimulation pathways. This may identify further molecules of interest that should be monitored in more detail.

Monolayer culture is not ideal, however, as the cells are extracted from their 3-d matrix and allowed to proliferate. Despite the fact that they have been shown to retain their chondrocyte phenotype in monolayer for the duration of our study the cells will not be attached to the matrix in the same way as *in vivo* so will sense strain differently. There will also be altered exposure to the cytokines due to the lack of matrix. The use of 3-d culture in alginate has been used in the study of chondrocytes. This provides a similar conformation to the *in vivo* situation so is a better model for the study of chondrocytes. The problem has been that, until recently the extraction of protein from this was difficult to achieve without exposing the cells to conditions that would alter their composition from that seen at the end of the experimental protocol. However, the development of this technique makes it an exciting prospect to be able to take our studies and assess their relevance and reproducibility within 3-d culture.

Investigation of the effects of NO on matrix regulation

These studies have shown that cytokine stimulation led to the decrease of aggrecan mRNA levels at 4 and 12 hours. However, investigation of the effects of NO on aggrecan mRNA levels within our system have failed to directly link this decrease to the increased iNOS production and activity. These have been carried out using an iNOS inhibitor and NO donor. The increasing of the number of replicates would be beneficial here, increasing the power of these results and allowing more confidence when changes are monitored.

The level of aggrecan mRNA was detected at 4 and 12 hours. Some literature suggests that to detect an effect would require looking at an earlier time point, so this would be of value to study. The use of some other available inhibitors, including 1400W or GW274150 may be of use. Similarly an NO donor from the increasing list of those commercially available that releases NO at a slower rate, such as glyco-SNAPs or NONOates may enhance these studies.

These studies began the process of investigation of the effects of NO on aggrecan mRNA levels using knockout mice, however time constraints and difficulties obtaining the mice allowed for only one experiment to be carried out. This would obviously need to be repeated in order to gain results of any significance that conclusions could be drawn from, but would be a worthwhile exercise in understanding the effects of NO on aggrecan levels.

Aggrecan mRNA levels were detected at 4 and 12 hours in these studies. This allows an understanding of what has happened to the mRNA levels at these time points. However, in order to show how these levels have been affected it would be necessary to monitor mRNA degradation or synthesis. This could be achieved through the use of pharmacological inhibitors of mRNA synthesis and degradatory pathways. It would also be beneficial to study the changes in aggrecan mRNA levels at different time-points. This would allow an understanding of how these levels alter over time.

These studies only show what is happening to the aggrecan mRNA levels. It would be necessary, therefore, to enhance this research by measuring the levels of aggrecan protein produced. The detection of aggrecan breakdown epitopes in the medium of cultured cells using specific antibodies may also be of interest.

Although aggrecan is the major proteoglycan in cartilage there are many other molecules present. It would therefore be of interest to monitor the effect of NO on several of these molecules such as collagen II or the proteoglycans, decorin and fibromodulin. The potential number quantity of work involved in this means that an initial study using a gene chip may allow the identification of certain molecules that are worthy of further study.

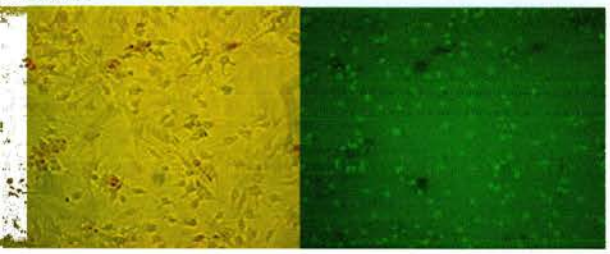
The other consideration is the conditions under which these cells are cultured. This is in light of recent evidence suggesting factors such as oxygen tension may influence the response of chondrocytes to molecules such as NO. It would therefore be of interest to determine if alteration of oxygen tension would influence the level of aggrecan mRNA within our system following proinflammatory cytokine stimulation.

The final consideration again stems from these studies being carried out in monolayer. The use of a model that more closely mimics the *in vivo* environment must be favoured. The repeating of these studies in 3-d culture would therefore be of considerable interest.

Appendix 1 – Acridine Orange Staining

I)

4hr ML



4hr Triton-x-100

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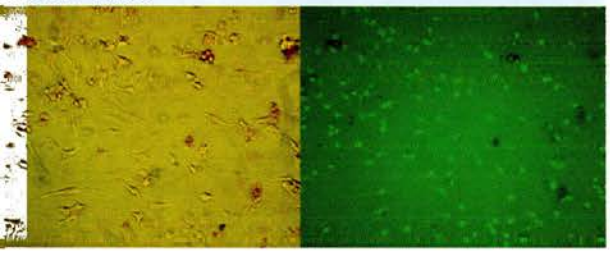

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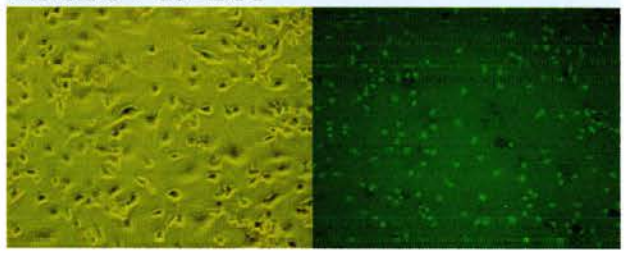

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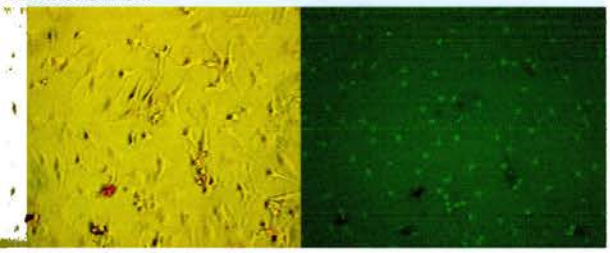

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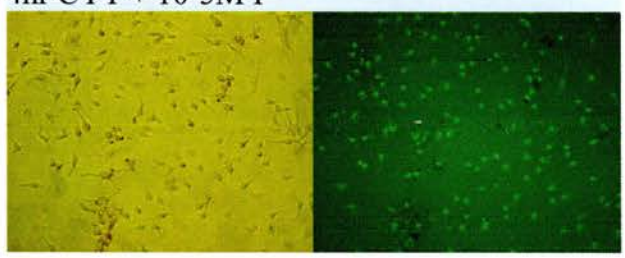

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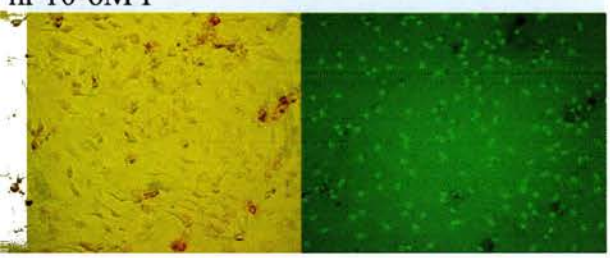

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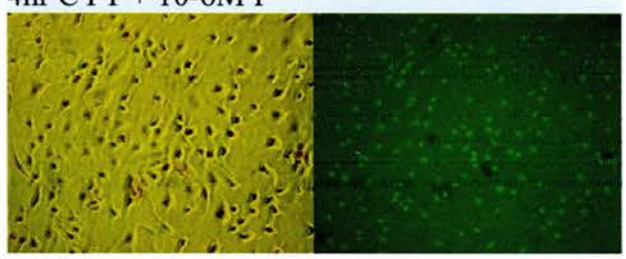
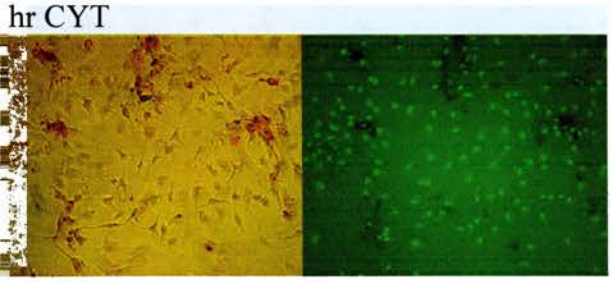


hr CYT



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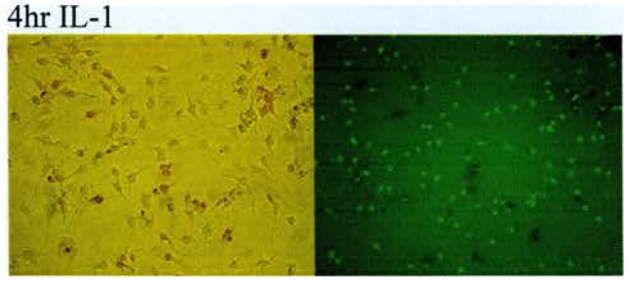
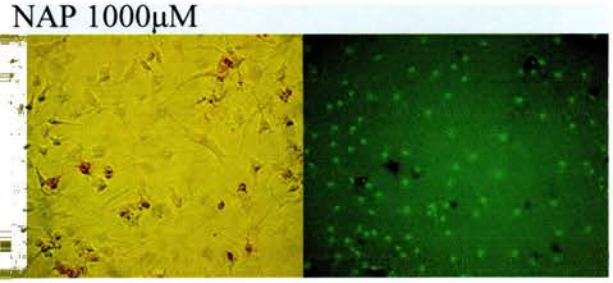
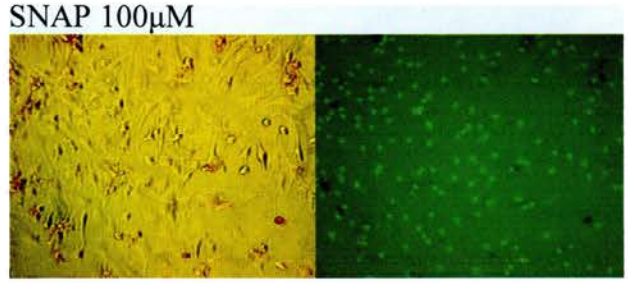


NAP 1000μM

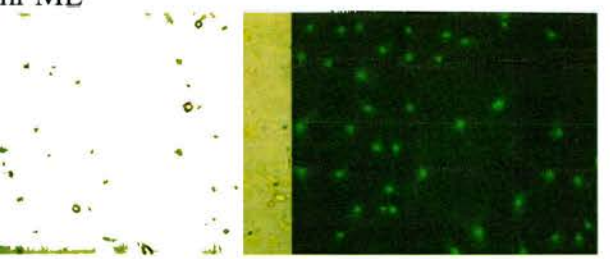


SNAP 100μM



II)

hr ML



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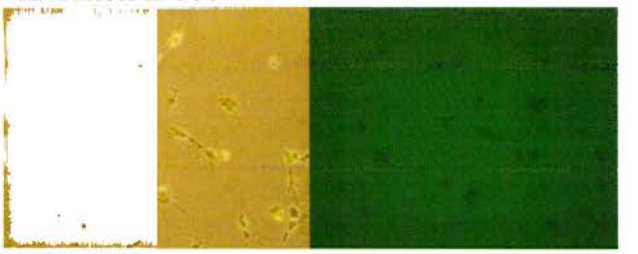
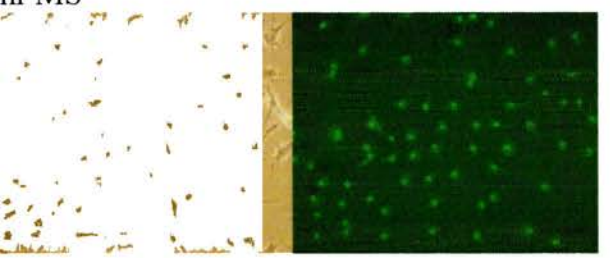


hr MS



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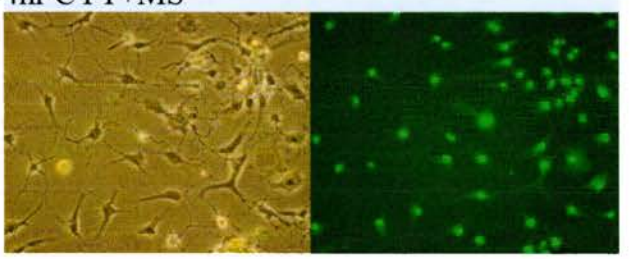
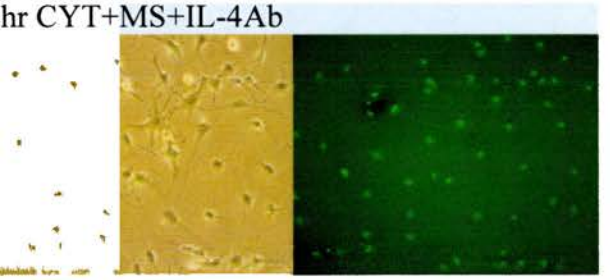
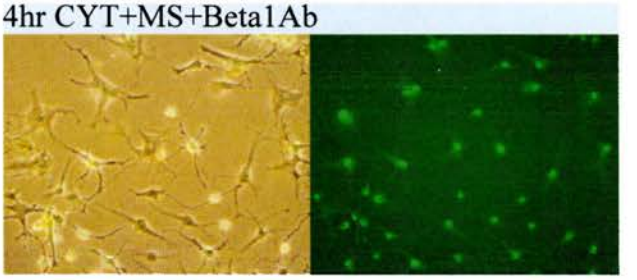


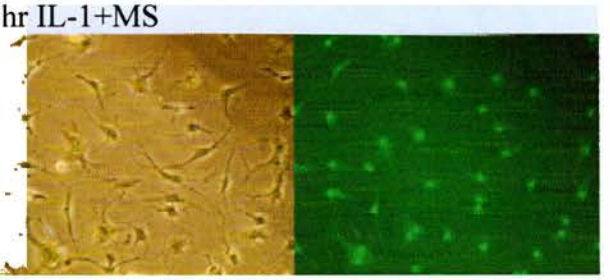
hr CYT+MS+IL-4Ab



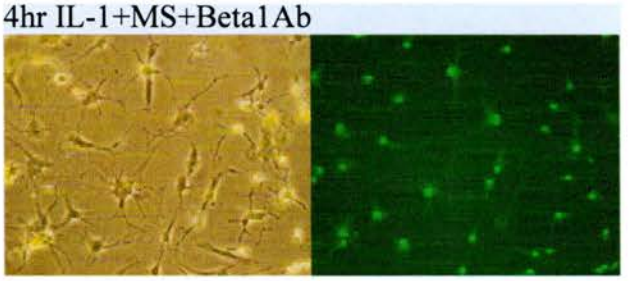
4hr CYT+MS+Beta1Ab



hr IL-1+MS

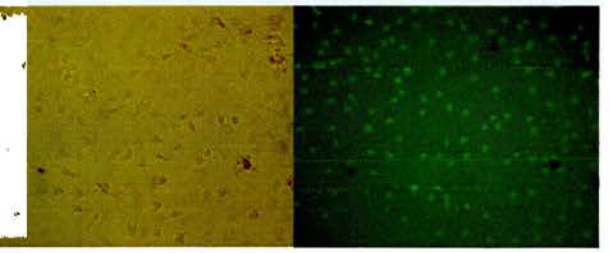


4hr IL-1+MS+Beta1Ab



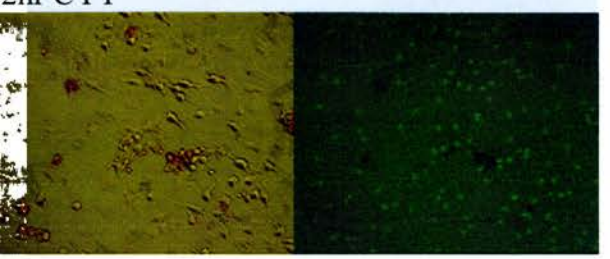
III)

2hr ML

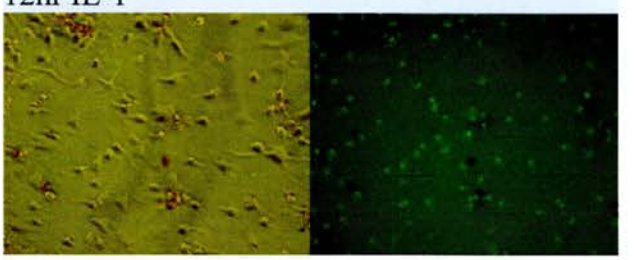


12hr Triton-x-100

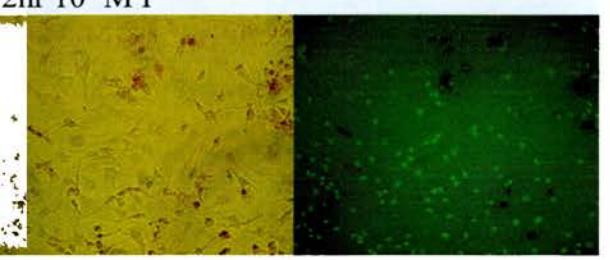
2hr CYT



12hr IL-1



2hr 10^{-4}M I



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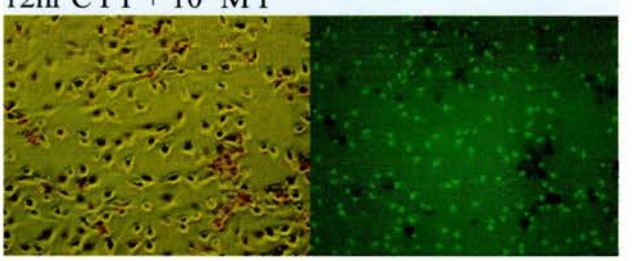

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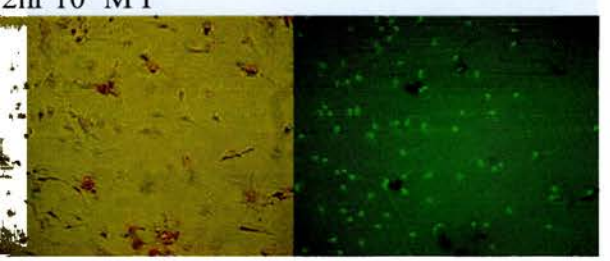

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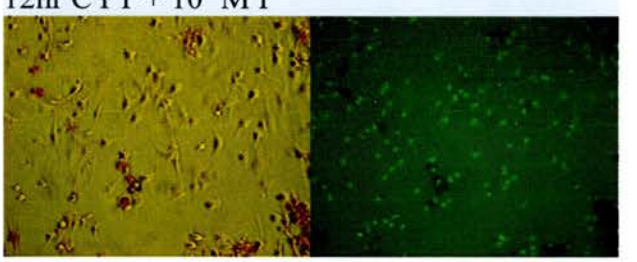

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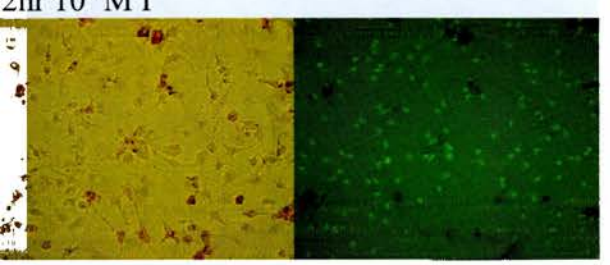
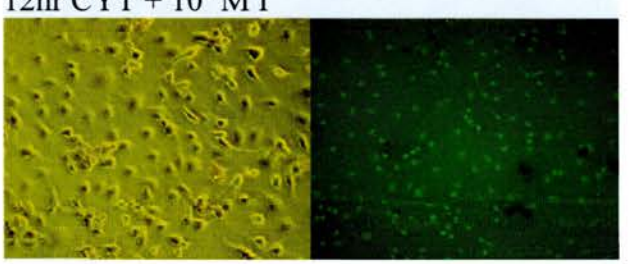
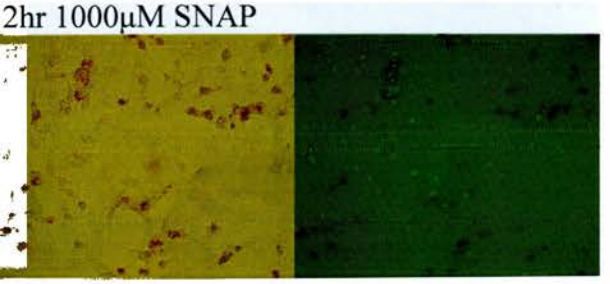


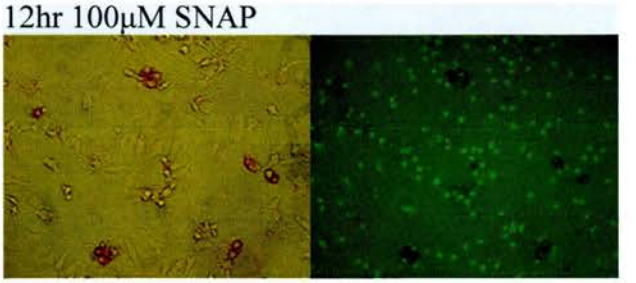
12hr CYT + 10^{-6}M I



2hr 1000μM SNAP



12hr 100μM SNAP



IV)

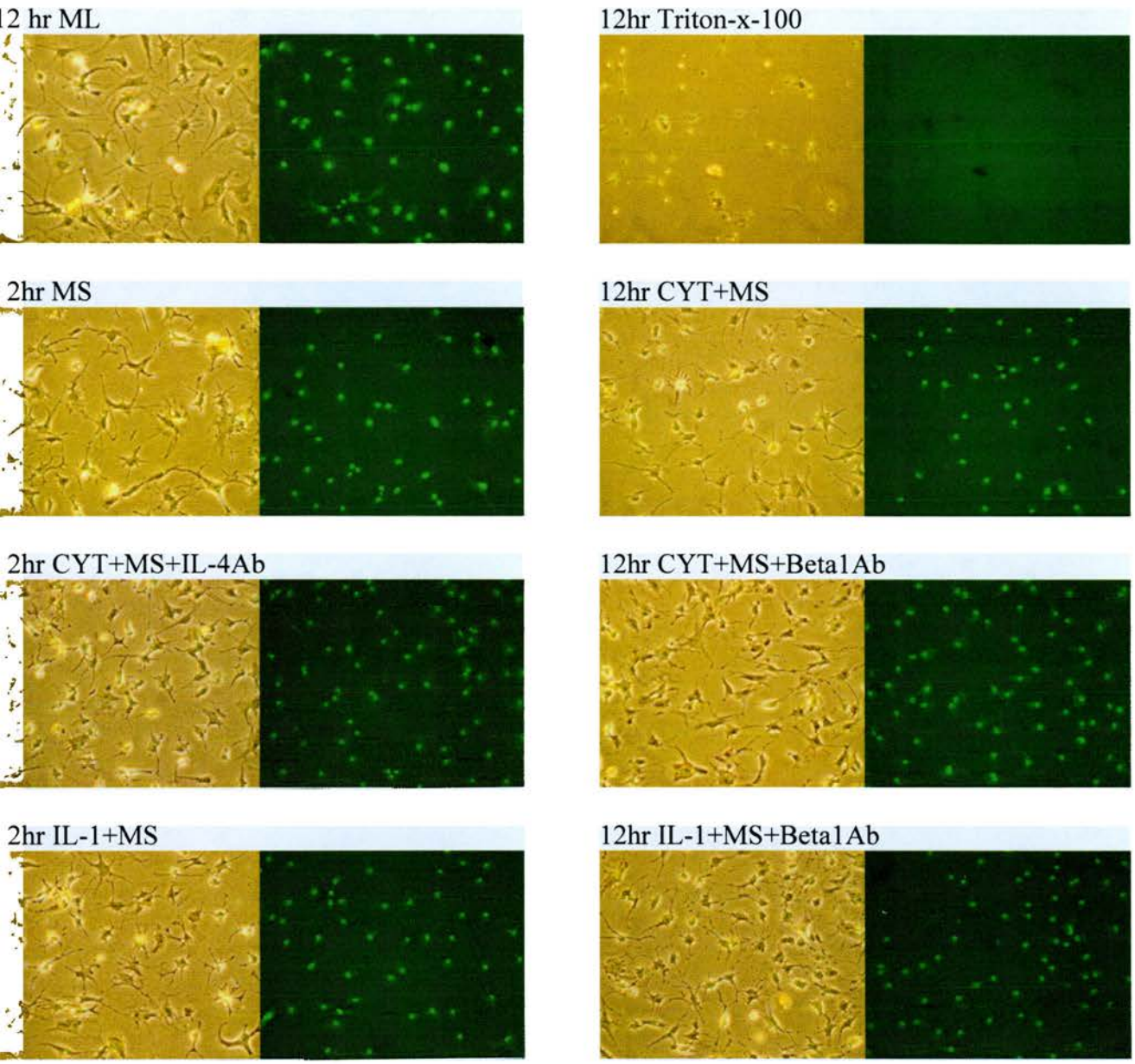
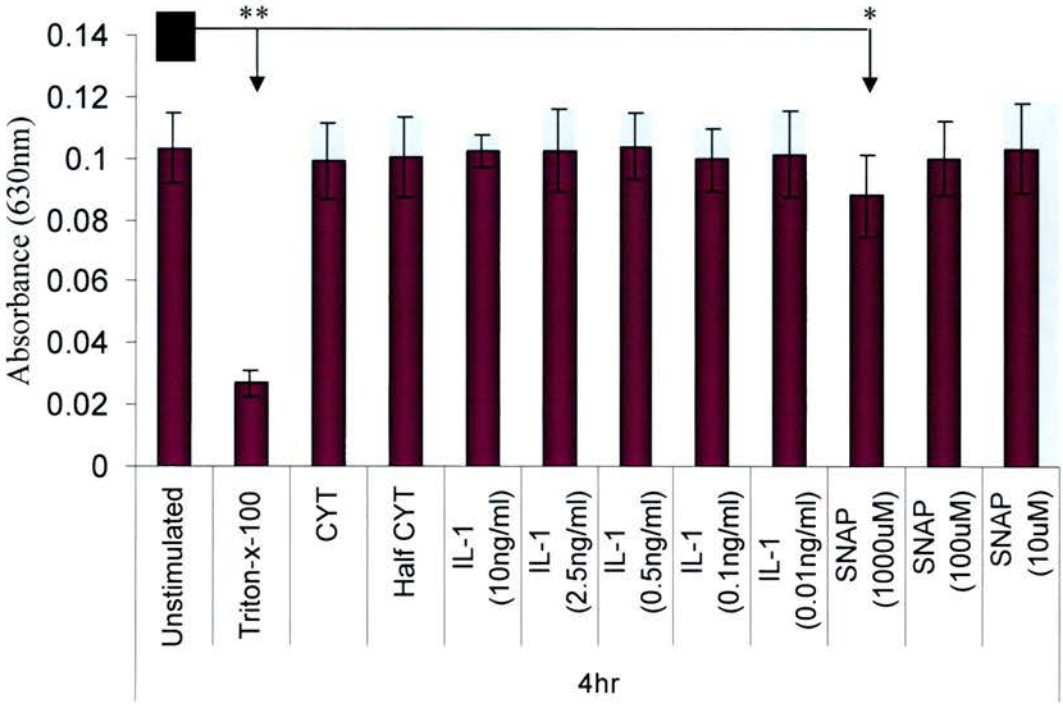


Figure 7.1 – Acridine orange cell viability studies using experimental conditions following 4 hour (I and II) and 12 hour (III and IV) incubation. CYT=Cytokine cocktail stimulation. MS=Mechanical stimulation. Beta1Ab=β1 integrin function blocking antibody (1μg/ml). IL-4Ab=IL-4 neutralising antibody (1μg/ml). I=Inhibitor AR-C102222 at 10^{-4}, 10^{-5} and 10^{-6}M. n=1

Appendix 2 – MTT Assays

(I)



(II)

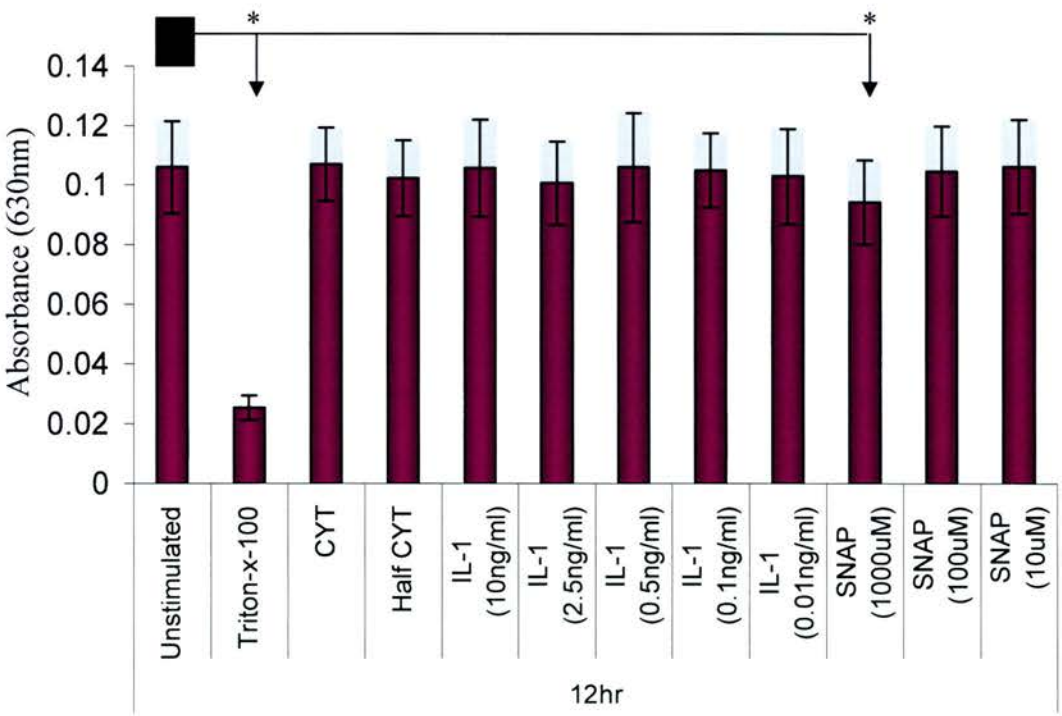
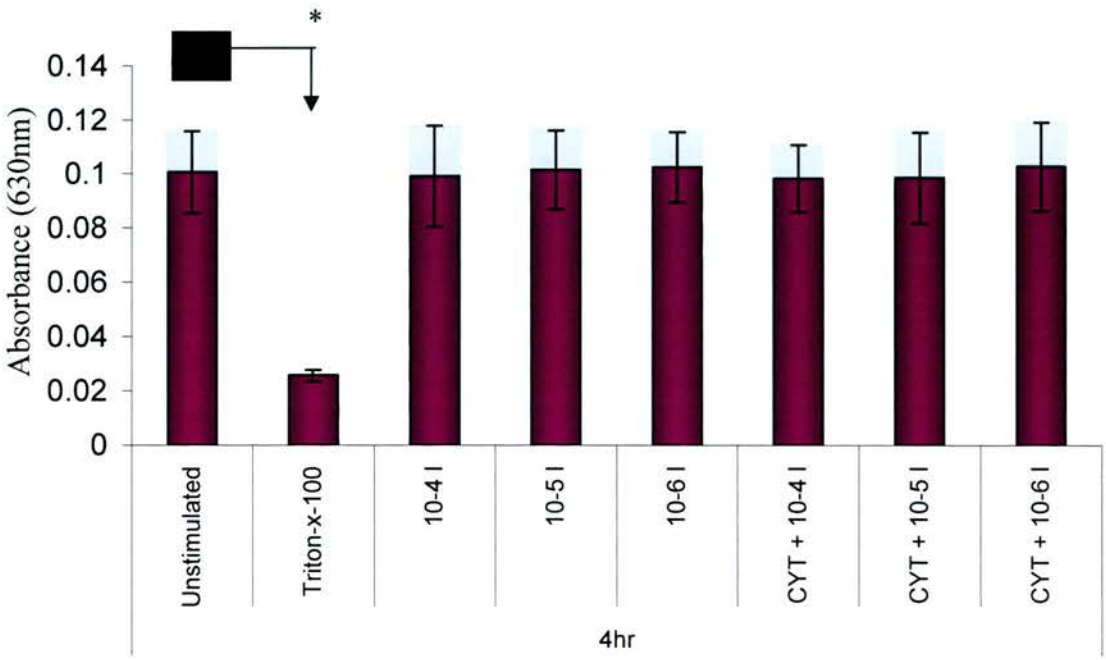


Figure 7.3 – MTT assays looking at cell viability under experimental conditions at (I) 4 hours and (II) 12 hours. CYT=Cytokine cocktail stimulation. n=3. *$p<0.05$ **$p<0.01$ (paired t test used).

(I)



(II)

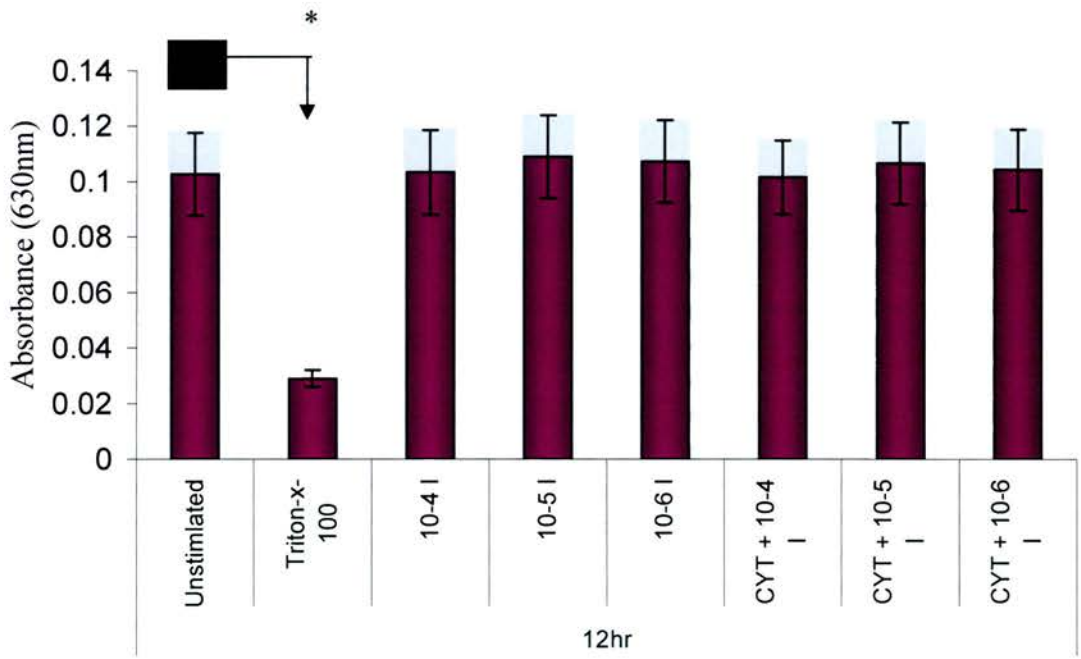


Figure 7.4 - MTT assays looking at cell viability under experimental conditions at (I) 4 hours and (II) 12 hours. I=Inhibitor AR-C102222 at 10^{-4}, 10^{-5} and 10^{-6}M. CYT=Cytokine cocktail stimulation. n=3. *$p<0.05$ (paired t test used).

Appendix 3 – Sample details

Expermental set	Date	Sample number	Collins OA Grade	Days in monolayer	Additional information
CYT+MS	22/06/03	DS031c	TA II	9	
	27/06/03	DS037c	TA I	10	
	28/06/03	DS037c	FA II	11	
	24/05/03	DS008/9c		10	
	17/06/03	14038	TA II	11	
	06/12/02	166WGH373c			
CYT+MS+β1Ab	22/03/04	14080	FA II	10	66 year old Female
	08/03/04	DS206c	FA II	11	
	10/02/04	DS184c	FA I	11	
	14/02/04	14077	TA II	8	70 year old Male
CYT+MS+IL-4Ab (monoclonal)	18/04/04	DS248c	TA II	11	
	03/10/03	DS104c	TA I	9	
	23/09/03	DS087c	FA I	11	
	11/11/03	DS126c	FA I	12	
	29/03/04	14081		11	72 year old Female
CYT+MS+IL-4Ab (polyclonal)	03/09/03	DS079c	TA III		
	05/09/03	14056	FA II		54 year old Female
IL-1 concentration	21/11/03	DS130c	FA II	10	
	21/05/04	14089	TA	9	73 year old Male
	16/04/04	DS248c	TA II	11	
	18/04/04	14085	FA II	9	53 year old Female
IL-1 complete	18/04/04	14085	FA II	9	53 year old Female
	06/04/04	DS242c	FA II	7	
	09/04/04	DS230c	FA II	10	
Inhibitor	06/05/03	DS002/2c		10	
	24/05/03	DS004/8c		10	
	24/05/03	DS008/9c		10	
	06/12/02	166WGH373c			
	17/06/03	14038	TA II	11	
	18/04/04	DS248c	TA II	11	
	11/05/04	14087	FA	11	56 year old Female
	21/05/04	14089	FA	9	73 year old Male
SNAP	28/06/03	DS037c	FA II	11	
	24/05/03	DS004/8c		10	
	24/05/03	DS008/9c		10	
MTT	24/05/04	DS274c	TA II		
	31/08/04	DS335c	FA		76 year old Male
	03/09/04	14099	TA		72 year old Male
Acridine Orange	05/09/04	14099	FA	9	72 year old Male
	28/09/04	14101	TA	11	72 year old Male

Table 7.1 – Sample details for experimental sets. CYT=Cytokine cocktail stimulation. MS=mechanical stimulation. β1Ab=β1 integrin function blocking antibody. IL-4Ab=IL-4 neutralising antibody. Inhibitor=AR-C102222. SNAP=NO donor. MTT and Acridine orange = cell viability. FA=fibial abnormal. TA=Tibial abnormal.

Appendix 4 – Published Abstracts

Accelerated proteoglycan synthesis in human chondrocyte cell lines following cyclic mechanical strain is associated with inhibition of neuronal nitric oxide synthase. *Rheumatology 41, Abstracts supplement 1, p125 abstract number 351 (2002)*

A novel highly selective quinazolinamine inhibitor of iNOS inhibits nitrite production, but not iNOS protein, in cytokine stimulated articular chondrocytes from patients with osteoarthritis. *Osteoarthritis and Cartilage 11, Supplement A, S100, abstract number P280 (2003).*

Cyclic mechanical stimulation decreases inducible nitric oxide synthase levels through a β1 integrin independent pathway in primary osteoarthritic human articular chondrocytes. *Osteoarthritis and Cartilage 12, Supplement B, S107, abstract number P265 (2004).*

References

1. Abu-Soud HM, Yoho LL, Stuehr DJ (1994). Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and interdomain electron transfer. *J Biol Chem* 269:32047-32050.

2. Adak S, Ghosh S, Abu-Soud HM, Stuehr DJ (1999). Role of reductase domain cluster 1 acidic residues in neuronal nitric-oxide synthase. Characterization of the FMN-FREE enzyme. *J Biol Chem* 274:22313-22320.

3. Adak S, Stuehr DJ (2001). A proximal tryptophan in NO synthase controls activity by a novel mechanism. *J Inorg Biochem* 83:301-308.

4. Agarwal S, Long P, Gassner R, Piesco NP, Buckley MJ (2001). Cyclic tensile strain suppresses catabolic effects of interleukin-1beta in fibrochondrocytes from the temporomandibular joint. *Arthritis Rheum* 44:608-617.

5. Agarwal S, Long P, Seyedain A, Piesco N, Shree A, Gassner R (2003). A central role for the nuclear factor-kappaB pathway in anti-inflammatory and proinflammatory actions of mechanical strain. *FASEB J* 17:899-901.

6. Agarwal S, Deschner J, Long P, Verma A, Hofman C, Evans CH, Piesco N (2004). Role of NF-kappaB transcription factors in antiinflammatory and proinflammatory actions of mechanical signals. *Arthritis Rheum* 50:3541-3548.

7. Ahluwalia J, Tinker A, Clapp LH, Duchen MR, Abramov AY, Pope S, Nobles M, Segal AW (2004). The large-conductance Ca2+-activated K+ channel is essential for innate immunity. *Nature* 427:853-858.

8. Aigner T, Dudhia J (1997). Phenotypic modulation of chondrocytes as a potential therapeutic target in osteoarthritis: a hypothesis. *Ann Rheum Dis* 56:287-291.

9. Aigner T, McKenna L (2002a). Molecular pathology and pathobiology of osteoarthritic cartilage. *Cell Mol Life Sci* 59:5-18.

10. Aigner T, Bartnik E, Zien A, Zimmer R (2002b). Functional genomics of osteoarthritis. *Pharmacogenomics* 3:635-650.

11. Aigner T, Kurz B, Fukui N, Sandell L (2002). Roles of chondrocytes in the pathogenesis of osteoarthritis. *Curr Opin Rheumatol* 14:578-584.

12. Akira S, Taga T, Kishimoto T (1993). Interleukin-6 in biology and medicine. *Adv Immunol* 54:1-78.

13. Albakri QA, Stuehr DJ (1996). Intracellular assembly of inducible NO synthase is limited by nitric oxide-mediated changes in heme insertion and availability. *J Biol Chem* 271:5414-5421.

14. Alderton WK, Cooper CE, Knowles RG (2001). Nitric oxide synthases: structure, function and inhibition. *Biochem J* 357:593-615.

15. Aldieri E, Orecchia S, Ghigo D, Bergandi L, Riganti C, Fubini B, Betta PG, Bosia A (2004). Simian virus 40 infection down-regulates the expression of nitric oxide synthase in human mesothelial cells. *Cancer Res* 64:4082-4084.

16. Alexopoulos LG, Haider MA, Vail TP, Guilak F (2003). Alterations in the mechanical properties of the human chondrocyte pericellular matrix with osteoarthritis. *J Biomech Eng* 125:323-333.

17. Amin AR, Attur M, Patel RN, Thakker GD, Marshall PJ, Rediske J, Stuchin SA, Patel IR, Abramson SB (1997). Superinduction of cyclooxygenase-2 activity in human osteoarthritis-affected cartilage. Influence of nitric oxide. *J Clin Invest* 99:1231-1237.

18. Anderson JJ, Felson DT (1988). Factors associated with osteoarthritis of the knee in the first national Health and Nutrition Examination Survey (HANES I). Evidence for an association with overweight, race, and physical demands of work. *Am J Epidemiol* 128:179-189.

19. Aplin AE, Howe A, Alahari SK, Juliano RL (1998). Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharmacol Rev* 50:197-263.

20. Armour KJ, Armour KE, van't Hof RJ, Reid DM, Wei XQ, Liew FY, Ralston SH (2001). Activation of the inducible nitric oxide synthase pathway contributes to inflammation-induced osteoporosis by suppressing bone formation and causing osteoblast apoptosis. *Arthritis Rheum* 44:2790-2796.

21. Armstrong CG, Mow VC (1982). Variations in the intrinsic mechanical properties of human articular cartilage with age, degeneration, and water content. *J Bone Joint Surg Am* 64:88-94.

22. Arner EC, Pratta MA (1991). Modulation of interleukin-1-induced alterations in cartilage proteoglycan metabolism by activation of protein kinase C. *Arthritis Rheum* 34:1006-1013.

23. Arner EC, Tortorella MD (1995). Signal transduction through chondrocyte integrin receptors induces matrix metalloproteinase synthesis and synergizes with interleukin-1. *Arthritis Rheum* 38:1304-1314.

24. Askew SC, Butler AR, Flitney FW, Kemp GD, Megson IL (1995). Chemical mechanisms underlying the vasodilator and platelet anti-aggregating properties of S-nitroso-N-acetyl-DL-penicillamine and S-nitrosoglutathione. *Bioorg Med Chem* 3:1-9.

25. Attur MG, Dave M, Cipolletta C, Kang P, Goldring MB, Patel IR, Abramson SB, Amin AR (2000). Reversal of autocrine and paracrine effects of interleukin 1 (IL-1) in human arthritis by type II IL-1 decoy receptor. Potential for pharmacological intervention. *J Biol Chem* 275:40307-40315.

26. Attur MG, Dave MN, Leung MY, Cipolletta C, Meseck M, Woo SL, Amin AR (2002). Functional genomic analysis of type II IL-1beta decoy receptor: potential for gene therapy in human arthritis and inflammation. *J Immunol* 168:2001-2010.

27. Badger AM, Griswold DE, Kapadia R, Blake S, Swift BA, Hoffman SJ, Stroup GB, Webb E, Rieman DJ, Gowen M, Boehm JC, Adams JL, Lee JC (2000). Disease-modifying activity of SB 242235, a selective inhibitor of p38 mitogen-activated protein kinase, in rat adjuvant-induced arthritis. *Arthritis Rheum* 43:175-183.

28. Banner DW, D'Arcy A, Janes W, Gentz R, Schoenfeld HJ, Broger C, Loetscher H, Lesslauer W (1993). Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell* 73:431-445.

29. Behrens F, Kraft EL, Oegema TR, Jr. (1989). Biochemical changes in articular cartilage after joint immobilization by casting or external fixation. *J Orthop Res* 7:335-343.

30. Biel M, Zong X, Ludwig A, Sautter A, Hofmann F (1999). Structure and function of cyclic nucleotide-gated channels. *Rev Physiol Biochem Pharmacol* 135:151-171.

31. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chen J, Van Wart H, Poole AR (1997). Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J Clin Invest* 99:1534-1545.

32. Bird JL, May S, Bayliss MT (2000). Nitric oxide inhibits aggrecan degradation in explant cultures of equine articular cartilage. *Equine Vet J* 32:133-139.

33. Bird TA, Schule HD, Delaney PB, Sims JE, Thoma B, Dower SK (1992). Evidence that MAP (mitogen-activated protein) kinase activation may be a necessary but not sufficient signal for a restricted subset of responses in IL-1-treated epidermoid cells. *Cytokine* 4:429-440.

34. Blanco FJ, Ochs RL, Schwarz H, Lotz M (1995). Chondrocyte apoptosis induced by nitric oxide. *Am J Pathol* 146:75-85.

35. Bland-Ward PA, Moore PK (1995). 7-Nitro indazole derivatives are potent inhibitors of brain, endothelium and inducible isoforms of nitric oxide synthase. *Life Sci* 57:L131-L135.

36. Block JA, Inerot SE, Gitelis S, Kimura JH (1991). The effects of long term monolayer culture on the proteoglycan phenotype of a clonal population of mature human malignant chondrocytes. *Connect Tissue Res* 26:295-313.

37. Bogdan C (2001). Nitric oxide and the regulation of gene expression. *Trends Cell Biol* 11:66-75.

38. Boisclair YR, Wang J, Shi J, Hurst KR, Ooi GT (2000). Role of the suppressor of cytokine signaling-3 in mediating the inhibitory effects of interleukin-1beta on the growth hormone-dependent transcription of the acid-labile subunit gene in liver cells. *J Biol Chem* 275:3841-3847.

39. Bommel HM, Reif A, Frohlich LG, Frey A, Hofmann H, Marecak DM, Groehn V, Kotsonis P, La M, Koster S, Meinecke M, Bernhardt M, Weeger M, Ghisla S, Prestwich GD, Pfleiderer W, Schmidt HH (1998). Anti-pterins as tools to characterize the function of tetrahydrobiopterin in NO synthase. *J Biol Chem* 273:33142-33149.

40. Bonassar LJ, Sandy JD, Lark MW, Plaas AH, Frank EH, Grodzinsky AJ (1997). Inhibition of cartilage degradation and changes in physical properties induced by IL-1beta and retinoic acid using matrix metalloproteinase inhibitors. *Arch Biochem Biophys* 344:404-412.

41. Bredt DS, Snyder SH (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci U S A* 87:682-685.

42. Bresnihan B, Alvaro-Gracia JM, Cobby M, Doherty M, Domljan Z, Emery P, Nuki G, Pavelka K, Rau R, Rozman B, Watt I, Williams B, Aitchison R, McCabe D, Musikic P (1998). Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. *Arthritis Rheum* 41:2196-2204.

43. Brown EJ, Frazier WA (2001). Integrin-associated protein (CD47) and its ligands. *Trends Cell Biol* 11:130-135.

44. Brown PD, Benya PD (1988). Alterations in chondrocyte cytoskeletal architecture during phenotypic modulation by retinoic acid and dihydrocytochalasin B-induced reexpression. *J Cell Biol* 106:171-179.

45. Brown RA, Jones KL (1990). The synthesis and accumulation of fibronectin by human articular cartilage. *J Rheumatol* 17:65-72.

46. Brown RT, Ades IZ, Nordan RP (1995). An acute phase response factor/NF-kappa B site downstream of the junB gene that mediates responsiveness to interleukin-6 in a murine plasmacytoma. *J Biol Chem* 270:31129-31135.

47. Brown TD (2000). Techniques for mechanical stimulation of cells in vitro: a review. *J Biomech* 33:3-14.

48. Browning JA, Wilkins RJ (2002). The effect of intracellular alkalinisation on intracellular Ca(2+) homeostasis in a human chondrocyte cell line. *Pflugers Arch* 444:744-751.

49. Browning JA, Saunders K, Urban JP, Wilkins RJ (2004). The influence and interactions of hydrostatic and osmotic pressures on the intracellular milieu of chondrocytes. *Biorheology* 41:299-308.

50. Burton-Wurster N, Butler M, Harter S, Colombo C, Quintavalla J, Swartzendurber D, Arsenis C, Lust G (1986). Presence of fibronectin in articular cartilage in two animal models of osteoarthritis. *J Rheumatol* 13:175-182.

51. Burton-Wurster N, Borden C, Lust G, Macleod JN (1998). Expression of the (V+C)- fibronectin isoform is tightly linked to the presence of a cartilaginous matrix. *Matrix Biol* 17:193-203.

52. Bush PG, Hall AC (2003). The volume and morphology of chondrocytes within non-degenerate and degenerate human articular cartilage. *Osteoarthritis Cartilage* 11:242-251.

53. Butler DM, Malfait AM, Mason LJ, Warden PJ, Kollias G, Maini RN, Feldmann M, Brennan FM (1997). DBA/1 mice expressing the human TNF-alpha transgene develop a severe, erosive arthritis: characterization of the cytokine cascade and cellular composition. *J Immunol* 159:2867-2876.

54. Butler WJ, Hawthorne VM, Mikkelsen WM, Carman WJ, Bouthillier DL, Lamphiear DE, Kazi IU (1988). Prevalence of radiologically defined osteoarthritis in the finger and wrist joints of adult residents of Tecumseh, Michigan, 1962-65. *J Clin Epidemiol* 41:467-473.

55. Cai L, Suboc P, Hogue DA, Fei DT, Filvaroff EH (2002). Interleukin 17 induced nitric oxide suppresses matrix synthesis and protects cartilage from matrix breakdown. *J Rheumatol* 29:1725-1736.

56. Cake MA, Appleyard RC, Read RA, Ghosh P, Swain MV, Murrell GC (2003). Topical administration of the nitric oxide donor glyceryl trinitrate modifies the structural and biomechanical properties of ovine articular cartilage. *Osteoarthritis Cartilage* 11:872-878.

57. Cao M, Westerhausen-Larson A, Niyibizi C, Kavalkovich K, Georgescu HI, Rizzo CF, Hebda PA, Stefanovic-Racic M, Evans CH (1997). Nitric oxide inhibits the synthesis of type-II collagen without altering Col2A1 mRNA abundance: prolyl hydroxylase as a possible target. *Biochem J* 324 (Pt 1):305-310.

58. Caput D, Beutler B, Hartog K, Thayer R, Brown-Shimer S, Cerami A (1986). Identification of a common nucleotide sequence in the 3'-untranslated region of

mRNA molecules specifying inflammatory mediators. *Proc Natl Acad Sci U S A* 83:1670-1674.

59. Carlo MD, Jr., Loeser RF (2003). Increased oxidative stress with aging reduces chondrocyte survival: correlation with intracellular glutathione levels. *Arthritis Rheum* 48:3419-3430.

60. Carmichael GG, McMaster GK (1980). The analysis of nucleic acids in gels using glyoxal and acridine orange. *Methods Enzymol* 65:380-391.

61. Carney SL, Muir H (1988). The structure and function of cartilage proteoglycans. *Physiol Rev* 68:858-910.

62. Chen CS, Ingber DE (1999). Tensegrity and mechanoregulation: from skeleton to cytoskeleton. *Osteoarthritis Cartilage* 7:81-94.

63. Chen PF, Tsai AL, Wu KK (1995). Cysteine 99 of endothelial nitric oxide synthase (NOS-III) is critical for tetrahydrobiopterin-dependent NOS-III stability and activity. *Biochem Biophys Res Commun* 215:1119-1129.

64. Chen Y, Panda K, Stuehr DJ (2002). Control of nitric oxide synthase dimer assembly by a heme-NO-dependent mechanism. *Biochemistry* 41:4618-4625.

65. Chen ZP, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, Kemp BE (1999). AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett* 443:285-289.

66. Chenevier-Gobeaux C, Morin-Robinet S, Lemarechal H, Poiraudeau S, Ekindjian JC, Borderie D (2004). Effects of pro- and anti-inflammatory cytokines and nitric oxide donors on hyaluronic acid synthesis by synovial cells from patients with rheumatoid arthritis. *Clin Sci (Lond)* 107:291-296.

67. Cheng JJ, Wung BS, Chao YJ, Wang DL (2001). Sequential activation of protein kinase C (PKC)-alpha and PKC-epsilon contributes to sustained Raf/ERK1/2 activation in endothelial cells under mechanical strain. *J Biol Chem* 276:31368-31375.

68. Chiquet M, Renedo AS, Huber F, Fluck M (2003). How do fibroblasts translate mechanical signals into changes in extracellular matrix production? *Matrix Biol* 22:73-80.

69. Chowdhury TT, Bader DL, Lee DA (2001). Dynamic compression inhibits the synthesis of nitric oxide and PGE(2) by IL-1beta-stimulated chondrocytes cultured in agarose constructs. *Biochem Biophys Res Commun* 285:1168-1174.

70. Chowdhury TT, Bader DL, Lee DA (2003). Dynamic compression counteracts IL-1 beta-induced release of nitric oxide and PGE2 by superficial zone chondrocytes cultured in agarose constructs. *Osteoarthritis Cartilage* 11:688-696.

71. Chowdhury TT, Salter DM, Bader DL, Lee DA (2004). Integrin-mediated mechanotransduction processes in TGFbeta-stimulated monolayer-expanded chondrocytes. *Biochem Biophys Res Commun* 318:873-881.

72. Clancy R, Leszczynska J, Amin A, Levartovsky D, Abramson SB (1995). Nitric oxide stimulates ADP ribosylation of actin in association with the inhibition of actin polymerization in human neutrophils. *J Leukoc Biol* 58:196-202.

73. Clancy R (1999). Nitric oxide alters chondrocyte function by disrupting cytoskeletal signaling complexes. *Osteoarthritis Cartilage* 7:399-400.

74. Clancy RM, Rediske J, Tang X, Nijher N, Frenkel S, Philips M, Abramson SB (1997). Outside-in signaling in the chondrocyte. Nitric oxide disrupts fibronectin-induced assembly of a subplasmalemmal actin/rho A/focal adhesion kinase signaling complex. *J Clin Invest* 100:1789-1796.

75. Clancy RM, Gomez PF, Abramson SB (2004). Nitric oxide sustains nuclear factor kappaB activation in cytokine-stimulated chondrocytes. *Osteoarthritis Cartilage* 12:552-558.

76. Coleman JW (2001). Nitric oxide in immunity and inflammation. *Int Immunopharmacol* 1:1397-1406.

77. Collins DH, McElligott TF (1960). Sulphate (35SO4) uptake by chondrocytes in relation to histological changes in osteoarthritic human articular cartilage. *Ann Rheum Dis* 19:318-330.

78. Colotta F, Re F, Muzio M, Bertini R, Polentarutti N, Sironi M, Giri JG, Dower SK, Sims JE, Mantovani A (1993). Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science* 261:472-475.

79. Connor JR, Manning PT, Settle SL, Moore WM, Jerome GM, Webber RK, Tjoeng FS, Currie MG (1995). Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. *Eur J Pharmacol* 273:15-24.

80. Conte A (2003). Physiologic pH changes modulate calcium ion dependence of brain nitric oxide synthase in Carassius auratus. *Biochim Biophys Acta* 1619:29-38.

81. Cooper CE (1999). Nitric oxide and iron proteins. *Biochim Biophys Acta* 1411:290-309.

82. Craig DH, Chapman SK, Daff S (2002). Calmodulin activates electron transfer through neuronal nitric-oxide synthase reductase domain by releasing an NADPH-dependent conformational lock. *J Biol Chem* 277:33987-33994.

83. Crane BR, Arvai AS, Ghosh DK, Wu C, Getzoff ED, Stuehr DJ, Tainer JA (1998). Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. *Science* 279:2121-2126.

84. Cuzzocrea S, Chatterjee PK, Mazzon E, McDonald MC, Dugo L, Di Paola R, Serraino I, Britti D, Caputi AP, Thiemermann C (2002). Beneficial effects of GW274150, a novel, potent and selective inhibitor of iNOS activity, in a rodent model of collagen-induced arthritis. *Eur J Pharmacol* 453:119-129.

85. Cuzzocrea S, Mazzon E, Dugo L, Genovese T, Di Paola R, Ruggeri Z, Vegeto E, Caputi AP, van de Loo FA, Puzzolo D, Maggi A (2003). Inducible nitric oxide synthase mediates bone loss in ovariectomized mice. *Endocrinology* 144:1098-1107.

86. D'Andrea P, Calabrese A, Capozzi I, Grandolfo M, Tonon R, Vittur F (2000). Intercellular Ca2+ waves in mechanically stimulated articular chondrocytes. *Biorheology* 37:75-83.

87. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA (1993). Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* 259:1739-1742.

88. Das P, Schurman DJ, Smith RL (1997). Nitric oxide and G proteins mediate the response of bovine articular chondrocytes to fluid-induced shear. *J Orthop Res* 15:87-93.

89. de Vera ME, Shapiro RA, Nussler AK, Mudgett JS, Simmons RL, Morris SM, Jr., Billiar TR, Geller DA (1996). Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: initial analysis of the human NOS2 promoter. *Proc Natl Acad Sci U S A* 93:1054-1059.

90. Dedhar S (2000). Cell-substrate interactions and signaling through ILK. *Curr Opin Cell Biol* 12:250-256.

91. Del CM, Jr., Loeser RF (2002). Nitric oxide-mediated chondrocyte cell death requires the generation of additional reactive oxygen species. *Arthritis Rheum* 46:394-403.

92. Dimmeler S, Lottspeich F, Brune B (1992). Nitric oxide causes ADP-ribosylation and inhibition of glyceraldehyde-3-phosphate dehydrogenase. *J Biol Chem* 267:16771-16774.

93. Dinerman JL, Steiner JP, Dawson TM, Dawson V, Snyder SH (1994). Cyclic nucleotide dependent phosphorylation of neuronal nitric oxide synthase inhibits catalytic activity. *Neuropharmacology* 33:1245-1251.

94. Doege KJ, Sasaki M, Kimura T, Yamada Y (1991). Complete coding sequence and deduced primary structure of the human cartilage large aggregating proteoglycan, aggrecan. Human-specific repeats, and additional alternatively spliced forms. *J Biol Chem* 266:894-902.

95. Duman RS, Terwilliger RZ, Nestler EJ (1991). Endogenous ADP-ribosylation in brain: initial characterization of substrate proteins. *J Neurochem* 57:2124-2132.

96. Edgell CJ, McDonald CC, Graham JB (1983). Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci U S A* 80:3734-3737.

97. Eissa NT, Strauss AJ, Haggerty CM, Choo EK, Chu SC, Moss J (1996). Alternative splicing of human inducible nitric-oxide synthase mRNA. tissue-specific regulation and induction by cytokines. *J Biol Chem* 271:27184-27187.

98. Eissa NT, Yuan JW, Haggerty CM, Choo EK, Palmer CD, Moss J (1998). Cloning and characterization of human inducible nitric oxide synthase splice variants: a domain, encoded by exons 8 and 9, is critical for dimerization. *Proc Natl Acad Sci U S A* 95:7625-7630.

99. Eliasson MJ, Blackshaw S, Schell MJ, Snyder SH (1997). Neuronal nitric oxide synthase alternatively spliced forms: prominent functional localizations in the brain. *Proc Natl Acad Sci U S A* 94:3396-3401.

100. Embry JJ, Knudson W (2003). G1 domain of aggrecan cointernalizes with hyaluronan via a CD44-mediated mechanism in bovine articular chondrocytes. *Arthritis Rheum* 48:3431-3441.

101. Enomoto-Iwamoto M, Iwamoto M, Nakashima K, Mukudai Y, Boettiger D, Pacifici M, Kurisu K, Suzuki F (1997). Involvement of alpha5beta1 integrin in matrix interactions and proliferation of chondrocytes. *J Bone Miner Res* 12:1124-1132.

102. Erb L, Liu J, Ockerhausen J, Kong Q, Garrad RC, Griffin K, Neal C, Krugh B, Santiago-Perez LI, Gonzalez FA, Gresham HD, Turner JT, Weisman GA (2001). An RGD sequence in the P2Y(2) receptor interacts with alpha(V)beta(3) integrins and is required for G(o)-mediated signal transduction. *J Cell Biol* 153:491-501.

103. Erickson GR, Alexopoulos LG, Guilak F (2001). Hyper-osmotic stress induces volume change and calcium transients in chondrocytes by transmembrane, phospholipid, and G-protein pathways. *J Biomech* 34:1527-1535.

104. Eyre DR, Dickson IR, Van Ness K (1988). Collagen cross-linking in human bone and articular cartilage. Age-related changes in the content of mature hydroxypyridinium residues. *Biochem J* 252:495-500.

105. Fanger GR, Gerwins P, Widmann C, Jarpe MB, Johnson GL (1997). MEKKs, GCKs, MLKs, PAKs, TAKs, and tpls: upstream regulators of the c-Jun amino-terminal kinases? *Curr Opin Genet Dev* 7:67-74.

106. Fanger GR, Widmann C, Porter AC, Sather S, Johnson GL, Vaillancourt RR (1998). 14-3-3 proteins interact with specific MEK kinases. *J Biol Chem* 273:3476-3483.

107. Fanning PJ, Emkey G, Smith RJ, Grodzinsky AJ, Szasz N, Trippel SB (2003). Mechanical regulation of mitogen-activated protein kinase signaling in articular cartilage. *J Biol Chem* 278:50940-50948.

108. Farrar MA, Schreiber RD (1993). The molecular cell biology of interferon-gamma and its receptor. *Annu Rev Immunol* 11:571-611.

109. Farrell AJ, Blake DR (1996). Nitric oxide. *Ann Rheum Dis* 55:7-20.

110. Fedorov R, Hartmann E, Ghosh DK, Schlichting I (2003). Structural basis for the specificity of the nitric-oxide synthase inhibitors W1400 and Nomega-propyl-L-Arg for the inducible and neuronal isoforms. *J Biol Chem* 278:45818-45825.

111. Felley-Bosco E, Bender FC, Courjault-Gautier F, Bron C, Quest AF (2000). Caveolin-1 down-regulates inducible nitric oxide synthase via the proteasome pathway in human colon carcinoma cells. *Proc Natl Acad Sci U S A* 97:14334-14339.

112. Feng X, Guo Z, Nourbakhsh M, Hauser H, Ganster R, Shao L, Geller DA (2002). Identification of a negative response element in the human inducible nitric-oxide synthase (hiNOS) promoter: The role of NF-kappa B-repressing factor (NRF) in basal repression of the hiNOS gene. *Proc Natl Acad Sci U S A* 99:14212-14217.

113. Fermor B, Weinberg JB, Pisetsky DS, Misukonis MA, Banes AJ, Guilak F (2001). The effects of static and intermittent compression on nitric oxide production in articular cartilage explants. *J Orthop Res* 19:729-737.

114. Fermor B, Weinberg JB, Pisetsky DS, Misukonis MA, Fink C, Guilak F (2002). Induction of cyclooxygenase-2 by mechanical stress through a nitric oxide-regulated pathway. *Osteoarthritis Cartilage* 10:792-798.

115. Fischmann TO, Hruza A, Niu XD, Fossetta JD, Lunn CA, Dolphin E, Prongay AJ, Reichert P, Lundell DJ, Narula SK, Weber PC (1999). Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. *Nat Struct Biol* 6:233-242.

116. Flam BR, Hartmann PJ, Harrell-Booth M, Solomonson LP, Eichler DC (2001). Caveolar localization of arginine regeneration enzymes, argininosuccinate synthase, and lyase, with endothelial nitric oxide synthase. *Nitric Oxide* 5:187-197.

117. Fountoulakis M, Zulauf M, Lustig A, Garotta G (1992). Stoichiometry of interaction between interferon gamma and its receptor. *Eur J Biochem* 208:781-787.

118. Frenkel SR, Clancy RM, Ricci JL, Di Cesare PE, Rediske JJ, Abramson SB (1996). Effects of nitric oxide on chondrocyte migration, adhesion, and cytoskeletal assembly. *Arthritis Rheum* 39:1905-1912.

119. Fujimoto TT, Katsutani S, Shimomura T, Fujimura K (2003). Thrombospondin-bound integrin-associated protein (CD47) physically and functionally modifies integrin alphaIIbbeta3 by its extracellular domain. *J Biol Chem* 278:26655-26665.

120. Fukuda S, Hashimoto N, Naritomi H, Nagata I, Nozaki K, Kondo S, Kurino M, Kikuchi H (2000). Prevention of rat cerebral aneurysm formation by inhibition of nitric oxide synthase. *Circulation* 101:2532-2538.

121. Gabriel SE, Crowson CS, Campion ME, O'Fallon WM (1997). Direct medical costs unique to people with arthritis. *J Rheumatol* 24:719-725.

122. Ganster RW, Taylor BS, Shao L, Geller DA (2001). Complex regulation of human inducible nitric oxide synthase gene transcription by Stat 1 and NF-kappa B. *Proc Natl Acad Sci U S A* 98:8638-8643.

123. Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJ, Knowles RG (1997). 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. *J Biol Chem* 272:4959-4963.

124. Gassner R, Buckley MJ, Georgescu H, Studer R, Stefanovich-Racic M, Piesco NP, Evans CH, Agarwal S (1999). Cyclic tensile stress exerts antiinflammatory actions on chondrocytes by inhibiting inducible nitric oxide synthase. *J Immunol* 163:2187-2192.

125. Gassner RJ, Buckley MJ, Studer RK, Evans CH, Agarwal S (2000). Interaction of strain and interleukin-1 in articular cartilage: effects on proteoglycan synthesis in chondrocytes. *Int J Oral Maxillofac Surg* 29:389-394.

126. Geller DA, Lowenstein CJ, Shapiro RA, Nussler AK, Di Silvio M, Wang SC, Nakayama DK, Simmons RL, Snyder SH, Billiar TR (1993). Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proc Natl Acad Sci U S A* 90:3491-3495.

127. Geng Y, Lotz M (1995). Increased intracellular Ca2+ selectively suppresses IL-1-induced NO production by reducing iNOS mRNA stability. *J Cell Biol* 129:1651-1657.

128. Ghosh DK, Stuehr DJ (1995). Macrophage NO synthase: characterization of isolated oxygenase and reductase domains reveals a head-to-head subunit interaction. *Biochemistry* 34:801-807.

129. Giancotti FG, Ruoslahti E (1999). Integrin signaling. *Science* 285:1028-1032.

130. Gilbert MS, Saad AH, Rupnow BA, Knox SJ (1996). Association of BCL-2 with membrane hyperpolarization and radioresistance. *J Cell Physiol* 168:114-122.

131. Gilkeson GS, Mudgett JS, Seldin MF, Ruiz P, Alexander AA, Misukonis MA, Pisetsky DS, Weinberg JB (1997). Clinical and serologic manifestations of autoimmune disease in MRL-lpr/lpr mice lacking nitric oxide synthase type 2. *J Exp Med* 186:365-373.

132. Goldring MB, Birkhead JR, Suen LF, Yamin R, Mizuno S, Glowacki J, Arbiser JL, Apperley JF (1994a). Interleukin-1 beta-modulated gene expression in immortalized human chondrocytes. *J Clin Invest* 94:2307-2316.

133. Goldring MB, Fukuo K, Birkhead JR, Dudek E, Sandell LJ (1994b). Transcriptional suppression by interleukin-1 and interferon-gamma of type II collagen gene expression in human chondrocytes. *J Cell Biochem* 54:85-99.

134. Goldring MB (1999). The role of cytokines as inflammatory mediators in osteoarthritis: lessons from animal models. *Connect Tissue Res* 40:1-11.

135. Goldring MB, Berenbaum F (1999). Human chondrocyte culture models for studying cyclooxygenase expression and prostaglandin regulation of collagen gene expression. *Osteoarthritis Cartilage* 7:386-388.

136. Goldring MB (2000). Osteoarthritis and cartilage: the role of cytokines. *Curr Rheumatol Rep* 2:459-465.

137. Gopalakrishna R, Chen ZH, Gundimeda U (1993). Nitric oxide and nitric oxide-generating agents induce a reversible inactivation of protein kinase C activity and phorbol ester binding. *J Biol Chem* 268:27180-27185.

138. Grabowski PS, Macpherson H, Ralston SH (1996). Nitric oxide production in cells derived from the human joint. *Br J Rheumatol* 35:207-212.

139. Grabowski PS, Wright PK, 't Hof RJ, Helfrich MH, Ohshima H, Ralston SH (1997). Immunolocalization of inducible nitric oxide synthase in synovium and cartilage in rheumatoid arthritis and osteoarthritis. *Br J Rheumatol* 36:651-655.

140. Graff RD, Lee GM (2003). Microplate live cell assay system for early events in mechanotransduction. *Anal Biochem* 318:181-186.

141. Gray ML, Pizzanelli AM, Lee RC, Grodzinsky AJ, Swann DA (1989). Kinetics of the chondrocyte biosynthetic response to compressive load and release. *Biochim Biophys Acta* 991:415-425.

142. Greenfeder SA, Nunes P, Kwee L, Labow M, Chizzonite RA, Ju G (1995). Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. *J Biol Chem* 270:13757-13765.

143. Gudi S, Huvar I, White CR, McKnight NL, Dusserre N, Boss GR, Frangos JA (2003). Rapid activation of Ras by fluid flow is mediated by Galpha(q) and Gbetagamma subunits of heterotrimeric G proteins in human endothelial cells. *Arterioscler Thromb Vasc Biol* 23:994-1000.

144. Guerne PA, Desgeorges A, Jaspar JM, Relic B, Peter R, Hoffmeyer P, Dayer JM (1999). Effects of IL-6 and its soluble receptor on proteoglycan synthesis and NO release by human articular chondrocytes: comparison with IL-1. Modulation by dexamethasone. *Matrix Biol* 18:253-260.

145. Hagemann C, Blank JL (2001). The ups and downs of MEK kinase interactions. *Cell Signal* 13:863-875.

146. Hall AC, Urban JP, Gehl KA (1991). The effects of hydrostatic pressure on matrix synthesis in articular cartilage. *J Orthop Res* 9:1-10.

147. Handy RL, Moore PK (1998). A comparison of the effects of L-NAME, 7-NI and L-NIL on carrageenan-induced hindpaw oedema and NOS activity. *Br J Pharmacol* 123:1119-1126.

148. Hannum CH, Wilcox CJ, Arend WP, Joslin FG, Dripps DJ, Heimdal PL, Armes LG, Sommer A, Eisenberg SP, Thompson RC (1990). Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. *Nature* 343:336-340.

149. Hara F, Fukuda K, Asada S, Matsukawa M, Hamanishi C (2001). Cyclic tensile stretch inhibition of nitric oxide release from osteoblast-like cells is both G protein and actin-dependent. *J Orthop Res* 19:126-131.

150. Hardingham T, Bayliss M (1990). Proteoglycans of articular cartilage: changes in aging and in joint disease. *Semin Arthritis Rheum* 20:12-33.

151. Hashimoto S, Ochs RL, Komiya S, Lotz M (1998). Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. *Arthritis Rheum* 41:1632-1638.

152. Hattori R, Sase K, Eizawa H, Kosuga K, Aoyama T, Inoue R, Sasayama S, Kawai C, Yui Y, Miyahara K, . (1994). Structure and function of nitric oxide synthases. *Int J Cardiol* 47:S71-S75.

153. Hauselmann HJ, Oppliger L, Michel BA, Stefanovic-Racic M, Evans CH (1994). Nitric oxide and proteoglycan biosynthesis by human articular chondrocytes in alginate culture. *FEBS Lett* 352:361-364.

154. Hauselmann HJ, Stefanovic-Racic M, Michel BA, Evans CH (1998). Differences in nitric oxide production by superficial and deep human articular chondrocytes: implications for proteoglycan turnover in inflammatory joint diseases. *J Immunol* 160:1444-1448.

155. Hayashi Y, Nishio M, Naito Y, Yokokura H, Nimura Y, Hidaka H, Watanabe Y (1999). Regulation of neuronal nitric-oxide synthase by calmodulin kinases. *J Biol Chem* 274:20597-20602.

156. Heinegard D, Oldberg A (1989). Structure and biology of cartilage and bone matrix noncollagenous macromolecules. *FASEB J* 3:2042-2051.

157. Hembry RM, Bagga MR, Reynolds JJ, Hamblen DL (1995). Immunolocalisation studies on six matrix metalloproteinases and their inhibitors, TIMP-1 and TIMP-2, in synovia from patients with osteo- and rheumatoid arthritis. *Ann Rheum Dis* 54:25-32.

158. Henrotin Y, Bassleer C, Collette J, Nusgens B, Franchimont P (1990). Radioimmunoassay for human type II collagen. *J Immunoassay* 11:555-578.

159. Henrotin YE, Zheng SX, Deby GP, Labasse AH, Crielaard JM, Reginster JY (1998). Nitric oxide downregulates interleukin 1beta (IL-1beta) stimulated IL-6, IL-8, and prostaglandin E2 production by human chondrocytes. *J Rheumatol* 25:1595-1601.

160. Henrotin YE, Zheng SX, Labasse AH, Deby GP, Crielaard JM, Reginster JY (2000). Modulation of human chondrocyte metabolism by recombinant human interferon. *Osteoarthritis Cartilage* 8:474-482.

161. Henrotin YE, Bruckner P, Pujol JP (2003). The role of reactive oxygen species in homeostasis and degradation of cartilage. *Osteoarthritis Cartilage* 11:747-755.

162. Hentze MW, Rouault TA, Harford JB, Klausner RD (1989). Oxidation-reduction and the molecular mechanism of a regulatory RNA-protein interaction. *Science* 244:357-359.

163. Hering TM (1999). Regulation of chondrocyte gene expression. *Front Biosci* 4:D743-D761.

164. Hibbs JB, Jr., Vavrin Z, Taintor RR (1987). L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J Immunol* 138:550-565.

165. Hibi M, Murakami M, Saito M, Hirano T, Taga T, Kishimoto T (1990). Molecular cloning and expression of an IL-6 signal transducer, gp130. *Cell* 63:1149-1157.

166. Hodge WA, Fijan RS, Carlson KL, Burgess RG, Harris WH, Mann RW (1986). Contact pressures in the human hip joint measured in vivo. *Proc Natl Acad Sci U S A* 83:2879-2883.

167. Hofmann F, Ammendola A, Schlossmann J (2000). Rising behind NO: cGMP-dependent protein kinases. *J Cell Sci* 113 (Pt 10):1671-1676.

168. Hollander AP, Pidoux I, Reiner A, Rorabeck C, Bourne R, Poole AR (1995). Damage to type II collagen in aging and osteoarthritis starts at the articular surface, originates around chondrocytes, and extends into the cartilage with progressive degeneration. *J Clin Invest* 96:2859-2869.

169. Homandberg GA, Meyers R, Xie DL (1992). Fibronectin fragments cause chondrolysis of bovine articular cartilage slices in culture. *J Biol Chem* 267:3597-3604.

170. Honda K, Ohno S, Tanimoto K, Ijuin C, Tanaka N, Doi T, Kato Y, Tanne K (2000). The effects of high magnitude cyclic tensile load on cartilage matrix metabolism in cultured chondrocytes. *Eur J Cell Biol* 79:601-609.

171. Horton WE, Jr., Udo I, Precht P, Balakir R, Hasty K (1998). Cytokine inducible matrix metalloproteinase expression in immortalized rat chondrocytes is independent of nitric oxide stimulation. *In Vitro Cell Dev Biol Anim* 34:378-384.

172. Howe AK, Juliano RL (1998). Distinct mechanisms mediate the initial and sustained phases of integrin-mediated activation of the Raf/MEK/mitogen-activated protein kinase cascade. *J Biol Chem* 273:27268-27274.

173. Hua LL, Zhao ML, Cosenza M, Kim MO, Huang H, Tanowitz HB, Brosnan CF, Lee SC (2002). Role of mitogen-activated protein kinases in inducible nitric oxide synthase and TNFalpha expression in human fetal astrocytes. *J Neuroimmunol* 126:180-189.

174. Huang H, Rose JL, Hoyt DG (2004). p38 Mitogen-activated protein kinase mediates synergistic induction of inducible nitric-oxide synthase by lipopolysaccharide and interferon-gamma through signal transducer and activator of transcription 1 Ser727 phosphorylation in murine aortic endothelial cells. *Mol Pharmacol* 66:302-311.

175. Huang WY, Valles S, Qwarnstrom EE (2001). Translocation of the IL-1 receptor to focal adhesions is regulated through the C-terminal end of the cytoplasmic domain. *Cell Biol Int* 25:309-317.

176. Hughes CE, Caterson B, Fosang AJ, Roughley PJ, Mort JS (1995). Monoclonal antibodies that specifically recognize neoepitope sequences generated by 'aggrecanase' and matrix metalloproteinase cleavage of aggrecan: application to catabolism in situ and in vitro. *Biochem J* 305 (Pt 3):799-804.

177. Hughes PE, Pfaff M (1998). Integrin affinity modulation. *Trends Cell Biol* 8:359-364.

178. Hung CT, Henshaw DR, Wang CC, Mauck RL, Raia F, Palmer G, Chao PH, Mow VC, Ratcliffe A, Valhmu WB (2000). Mitogen-activated protein kinase signaling in bovine articular chondrocytes in response to fluid flow does not require calcium mobilization. *J Biomech* 33:73-80.

179. Hynes RO (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69:11-25.

180. Iannone F, Corrado A, Grattagliano V, Cantatore FP, Patella V, Lapadula G (2001). [Phenotyping of chondrocytes from human osteoarthritic cartilage: chondrocyte expression of beta integrins and correlation with anatomic injury]. *Reumatismo* 53:122-130.

181. Ikenoue T, Trindade MC, Lee MS, Lin EY, Schurman DJ, Goodman SB, Smith RL (2003). Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure in vitro. *J Orthop Res* 21:110-116.

182. Ingram AJ, James L, Ly H, Thai K, Scholey JW (2000a). Stretch activation of jun N-terminal kinase/stress-activated protein kinase in mesangial cells. *Kidney Int* 58:1431-1439.

183. Ingram AJ, James L, Cai L, Thai K, Ly H, Scholey JW (2000b). NO inhibits stretch-induced MAPK activity by cytoskeletal disruption. *J Biol Chem* 275:40301-40306.

184. Ingram AJ, James L, Ly H, Thai K, Cai L, Scholey JW (2000c). Nitric oxide modulates stretch activation of mitogen-activated protein kinases in mesangial cells. *Kidney Int* 58:1067-1077.

185. Ingram AJ, James L, Thai K, Ly H, Cai L, Scholey JW (2000d). Nitric oxide modulates mechanical strain-induced activation of p38 MAPK in mesangial cells. *Am J Physiol Renal Physiol* 279:F243-F251.

186. Jin M, Emkey GR, Siparsky P, Trippel SB, Grodzinsky AJ (2003). Combined effects of dynamic tissue shear deformation and insulin-like growth factor I on

chondrocyte biosynthesis in cartilage explants. *Arch Biochem Biophys* 414:223-231.

187. Jobanputra P, Lin H, Jenkins K, Bavington C, Brennan FR, Nuki G, Salter DM, Godolphin JL (1996). Modulation of human chondrocyte integrins by inflammatory synovial fluid. *Arthritis Rheum* 39:1430-1432.

188. Johnson K, Jung A, Murphy A, Andreyev A, Dykens J, Terkeltaub R (2000). Mitochondrial oxidative phosphorylation is a downstream regulator of nitric oxide effects on chondrocyte matrix synthesis and mineralization. *Arthritis Rheum* 43:1560-1570.

189. Joosten LA, Lubberts E, Durez P, Helsen MM, Jacobs MJ, Goldman M, van den Berg WB (1997). Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis. Protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction. *Arthritis Rheum* 40:249-260.

190. Juedes MJ, Wogan GN (1996). Peroxynitrite-induced mutation spectra of pSP189 following replication in bacteria and in human cells. *Mutat Res* 349:51-61.

191. Juilfs DM, Soderling S, Burns F, Beavo JA (1999). Cyclic GMP as substrate and regulator of cyclic nucleotide phosphodiesterases (PDEs). *Rev Physiol Biochem Pharmacol* 135:67-104.

192. Julovi SM, Yasuda T, Shimizu M, Hiramitsu T, Nakamura T (2004). Inhibition of interleukin-1beta-stimulated production of matrix metalloproteinases by hyaluronan via CD44 in human articular cartilage. *Arthritis Rheum* 50:516-525.

193. Kaarniranta K, Elo MA, Sironen RK, Karjalainen HM, Helminen HJ, Lammi MJ (2003). Stress responses of mammalian cells to high hydrostatic pressure. *Biorheology* 40:87-92.

194. Kahle P, Saal JG, Schaudt K, Zacher J, Fritz P, Pawelec G (1992). Determination of cytokines in synovial fluids: correlation with diagnosis and histomorphological characteristics of synovial tissue. *Ann Rheum Dis* 51:731-734.

195. Kamada H, Masuda K, D'Souza AL, Lenz ME, Pietryla D, Otten L, Thonar EJ (2002). Age-related differences in the accumulation and size of hyaluronan in alginate culture. *Arch Biochem Biophys* 408:192-199.

196. Kamijo R, Shapiro D, Le J, Huang S, Aguet M, Vilcek J (1993). Generation of nitric oxide and induction of major histocompatibility complex class II antigen in macrophages from mice lacking the interferon gamma receptor. *Proc Natl Acad Sci U S A* 90:6626-6630.

197. Karandikar M, Xu S, Cobb MH (2000). MEKK1 binds raf-1 and the ERK2 cascade components. *J Biol Chem* 275:40120-40127.

198. Kavanaugh A (1998). Lipid profiles in patients with rheumatoid arthritis. *Ann Rheum Dis* 57:175.

199. Kempson GE, Muir H, Pollard C, Tuke M (1973). The tensile properties of the cartilage of human femoral condyles related to the content of collagen and glycosaminoglycans. *Biochim Biophys Acta* 297:456-472.

200. Kim SJ, Hwang SG, Shin DY, Kang SS, Chun JS (2002). p38 kinase regulates nitric oxide-induced apoptosis of articular chondrocytes by accumulating p53 via NFkappa B-dependent transcription and stabilization by serine 15 phosphorylation. *J Biol Chem* 277:33501-33508.

201. Kim SJ, Hwang SG, Kim IC, Chun JS (2003). Actin cytoskeletal architecture regulates nitric oxide-induced apoptosis, dedifferentiation, and cyclooxygenase-2 expression in articular chondrocytes via mitogen-activated protein kinase and protein kinase C pathways. *J Biol Chem* 278:42448-42456.

202. Kim SJ, Chun JS (2003). Protein kinase C alpha and zeta regulate nitric oxide-induced NF-kappa B activation that mediates cyclooxygenase-2 expression and apoptosis but not dedifferentiation in articular chondrocytes. *Biochem Biophys Res Commun* 303:206-211.

203. Kinugawa K, Shimizu T, Yao A, Kohmoto O, Serizawa T, Takahashi T (1997). Transcriptional regulation of inducible nitric oxide synthase in cultured neonatal rat cardiac myocytes. *Circ Res* 81:911-921.

204. Kiviranta I, Tammi M, Jurvelin J, Saamanen AM, Helminen HJ (1988). Moderate running exercise augments glycosaminoglycans and thickness of articular cartilage in the knee joint of young beagle dogs. *J Orthop Res* 6:188-195.

205. Klatt P, Schmidt K, Lehner D, Glatter O, Bachinger HP, Mayer B (1995). Structural analysis of porcine brain nitric oxide synthase reveals a role for tetrahydrobiopterin and L-arginine in the formation of an SDS-resistant dimer. *EMBO J* 14:3687-3695.

206. Knight MM, van de Breevaart BJ, Lee DA, van Osch GJ, Weinans H, Bader DL (2002). Cell and nucleus deformation in compressed chondrocyte-alginate constructs: temporal changes and calculation of cell modulus. *Biochim Biophys Acta* 1570:1-8.

207. Knudson CB (1993). Hyaluronan receptor-directed assembly of chondrocyte pericellular matrix. *J Cell Biol* 120:825-834.

208. Knudson W, Loeser RF (2002). CD44 and integrin matrix receptors participate in cartilage homeostasis. *Cell Mol Life Sci* 59:36-44.

209. Kolanus W, Seed B (1997). Integrins and inside-out signal transduction: converging signals from PKC and PIP3. *Curr Opin Cell Biol* 9:725-731.

210. Kolb JP (2000). Mechanisms involved in the pro- and anti-apoptotic role of NO in human leukemia. *Leukemia* 14:1685-1694.

211. Kone BC, Kuncewicz T, Zhang W, Yu ZY (2003). Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide. *Am J Physiol Renal Physiol* 285:F178-F190.

212. Kopp E, Medzhitov R, Carothers J, Xiao C, Douglas I, Janeway CA, Ghosh S (1999). ECSIT is an evolutionarily conserved intermediate in the Toll/IL-1 signal transduction pathway. *Genes Dev* 13:2059-2071.

213. Kordula T, Travis J (1996). The role of Stat and C/EBP transcription factors in the synergistic activation of rat serine protease inhibitor-3 gene by interleukin-6 and dexamethasone. *Biochem J* 313 (Pt 3):1019-1027.

214. Kroncke KD, Suschek CV, Kolb-Bachofen V (2000). Implications of inducible nitric oxide synthase expression and enzyme activity. *Antioxid Redox Signal* 2:585-605.

215. Kroncke KD, Carlberg C (2000). Inactivation of zinc finger transcription factors provides a mechanism for a gene regulatory role of nitric oxide. *FASEB J* 14:166-173.

216. Kroncke KD, Fehsel K, Suschek C, Kolb-Bachofen V (2001). Inducible nitric oxide synthase-derived nitric oxide in gene regulation, cell death and cell survival. *Int Immunopharmacol* 1:1407-1420.

217. Kroncke KD, Klotz LO, Suschek CV, Sies H (2002). Comparing nitrosative versus oxidative stress toward zinc finger-dependent transcription. Unique role for NO. *J Biol Chem* 277:13294-13301.

218. Kroncke KD (2003). Mechanisms and biological consequences of nitrosative stress. *Biol Chem* 384:1341.

219. Kuettner KE, Aydelotte MB, Thonar EJ (1991). Articular cartilage matrix and structure: a minireview. *J Rheumatol Suppl* 27:46-48.

220. Kuhn K, D'Lima DD, Hashimoto S, Lotz M (2004). Cell death in cartilage. *Osteoarthritis Cartilage* 12:1-16.

221. Kuncewicz T, Balakrishnan P, Snuggs MB, Kone BC (2001). Specific association of nitric oxide synthase-2 with Rac isoforms in activated murine macrophages. *Am J Physiol Renal Physiol* 281:F326-F336.

222. Lahiji K, Polotsky A, Hungerford DS, Frondoza CG (2004). Cyclic strain stimulates proliferative capacity, alpha2 and alpha5 integrin, gene marker expression by human articular chondrocytes propagated on flexible silicone membranes. *In Vitro Cell Dev Biol Anim* 40:138-142.

223. Lammi MJ, Inkinen R, Parkkinen JJ, Hakkinen T, Jortikka M, Nelimarkka LO, Jarvelainen HT, Tammi MI (1994). Expression of reduced amounts of structurally altered aggrecan in articular cartilage chondrocytes exposed to high hydrostatic pressure. *Biochem J* 304 (Pt 3):723-730.

224. Lander HM, Sehajpal P, Levine DM, Novogrodsky A (1993). Activation of human peripheral blood mononuclear cells by nitric oxide-generating compounds. *J Immunol* 150:1509-1516.

225. Langelier E, Suetterlin R, Hoemann CD, Aebi U, Buschmann MD (2000). The chondrocyte cytoskeleton in mature articular cartilage: structure and distribution of actin, tubulin, and vimentin filaments. *J Histochem Cytochem* 48:1307-1320.

226. Lark MW, Bayne EK, Flanagan J, Harper CF, Hoerrner LA, Hutchinson NI, Singer II, Donatelli SA, Weidner JR, Williams HR, Mumford RA, Lohmander LS (1997). Aggrecan degradation in human cartilage. Evidence for both matrix metalloproteinase and aggrecanase activity in normal, osteoarthritic, and rheumatoid joints. *J Clin Invest* 100:93-106.

227. Lawrence RC, Helmick CG, Arnett FC, Deyo RA, Felson DT, Giannini EH, Heyse SP, Hirsch R, Hochberg MC, Hunder GG, Liang MH, Pillemer SR, Steen VD, Wolfe F (1998). Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. *Arthritis Rheum* 41:778-799.

228. LeClair KP, Blanar MA, Sharp PA (1992). The p50 subunit of NF-kappa B associates with the NF-IL6 transcription factor. *Proc Natl Acad Sci U S A* 89:8145-8149.

229. Lee DA, Bader DL (1997). Compressive strains at physiological frequencies influence the metabolism of chondrocytes seeded in agarose. *J Orthop Res* 15:181-188.

230. Lee DA, Frean SP, Lees P, Bader DL (1998). Dynamic mechanical compression influences nitric oxide production by articular chondrocytes seeded in agarose. *Biochem Biophys Res Commun* 251:580-585.

231. Lee DA, Noguchi T, Frean SP, Lees P, Bader DL (2000). The influence of mechanical loading on isolated chondrocytes seeded in agarose constructs. *Biorheology* 37:149-161.

232. Lee HS, Millward-Sadler SJ, Wright MO, Nuki G, Salter DM (2000). Integrin and mechanosensitive ion channel-dependent tyrosine phosphorylation of focal adhesion proteins and beta-catenin in human articular chondrocytes after mechanical stimulation. *J Bone Miner Res* 15:1501-1509.

233. Lee HS, Millward-Sadler SJ, Wright MO, Nuki G, Al Jamal R, Salter DM (2002). Activation of Integrin-RACK1/PKCalpha signalling in human articular chondrocyte mechanotransduction. *Osteoarthritis Cartilage* 10:890-897.

234. Lee MS, Trindade MC, Ikenoue T, Schurman DJ, Goodman SB, Smith RL (2002). Effects of shear stress on nitric oxide and matrix protein gene expression in human osteoarthritic chondrocytes in vitro. *J Orthop Res* 20:556-561.

235. Lee MS, Ikenoue T, Trindade MC, Wong N, Goodman SB, Schurman DJ, Smith RL (2003a). Protective effects of intermittent hydrostatic pressure on osteoarthritic chondrocytes activated by bacterial endotoxin in vitro. *J Orthop Res* 21:117-122.

236. Lee MS, Trindade MC, Ikenoue T, Schurman DJ, Goodman SB, Smith RL (2003b). Intermittent hydrostatic pressure inhibits shear stress-induced nitric oxide release in human osteoarthritic chondrocytes in vitro. *J Rheumatol* 30:326-328.

237. Lepoivre M, Chenais B, Yapo A, Lemaire G, Thelander L, Tenu JP (1990). Alterations of ribonucleotide reductase activity following induction of the nitrite-generating pathway in adenocarcinoma cells. *J Biol Chem* 265:14143-14149.

238. Li H, Raman CS, Martasek P, Masters BS, Poulos TL (2001). Crystallographic studies on endothelial nitric oxide synthase complexed with nitric oxide and mechanism-based inhibitors. *Biochemistry* 40:5399-5406.

239. Li Z, Calzada MJ, Sipes JM, Cashel JA, Krutzsch HC, Annis DS, Mosher DF, Roberts DD (2002). Interactions of thrombospondins with alpha4beta1 integrin and CD47 differentially modulate T cell behavior. *J Cell Biol* 157:509-519.

240. Linn SC, Morelli PJ, Edry I, Cottongim SE, Szabo C, Salzman AL (1997). Transcriptional regulation of human inducible nitric oxide synthase gene in an intestinal epithelial cell line. *Am J Physiol* 272:G1499-G1508.

241. Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HS, Sucher NJ, Loscalzo J, Singel DJ, Stamler JS (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 364:626-632.

242. Little CB, Hughes CE, Curtis CL, Janusz MJ, Bohne R, Wang-Weigand S, Taiwo YO, Mitchell PG, Otterness IG, Flannery CR, Caterson B (2002). Matrix metalloproteinases are involved in C-terminal and interglobular domain processing of cartilage aggrecan in late stage cartilage degradation. *Matrix Biol* 21:271-288.

243. Liu GZ, Ishihara H, Osada R, Kimura T, Tsuji H (2001). Nitric oxide mediates the change of proteoglycan synthesis in the human lumbar intervertebral disc in response to hydrostatic pressure. *Spine* 26:134-141.

244. Liu RH, Jacob JR, Hotchkiss JH, Tennant BC (1993). Synthesis of nitric oxide and nitrosamine by immortalized woodchuck hepatocytes. *Carcinogenesis* 14:1609-1613.

245. Lo MY, Kim HT (2004). Chondrocyte apoptosis induced by hydrogen peroxide requires caspase activation but not mitochondrial pore transition. *J Orthop Res* 22:1120-1125.

246. Lo YY, Luo L, McCulloch CA, Cruz TF (1998). Requirements of focal adhesions and calcium fluxes for interleukin-1-induced ERK kinase activation and c-fos expression in fibroblasts. *J Biol Chem* 273:7059-7065.

247. Loeser RF, Carlson CS, McGee MP (1995). Expression of beta 1 integrins by cultured articular chondrocytes and in osteoarthritic cartilage. *Exp Cell Res* 217:248-257.

248. Loeser RF (1997). Growth factor regulation of chondrocyte integrins. Differential effects of insulin-like growth factor 1 and transforming growth factor beta on alpha 1 beta 1 integrin expression and chondrocyte adhesion to type VI collagen. *Arthritis Rheum* 40:270-276.

249. Loeser RF (2000). Chondrocyte integrin expression and function. *Biorheology* 37:109-116.

250. Loeser RF, Sadiev S, Tan L, Goldring MB (2000). Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for alpha1beta1 and alpha2beta1 integrins in mediating chondrocyte adhesion to types II and VI collagen. *Osteoarthritis Cartilage* 8:96-105.

251. Loeser RF, Carlson CS, Del Carlo M, Cole A (2002). Detection of nitrotyrosine in aging and osteoarthritic cartilage: Correlation of oxidative damage with the presence of interleukin-1beta and with chondrocyte resistance to insulin-like growth factor 1. *Arthritis Rheum* 46:2349-2357.

252. Loeser RF, Forsyth CB, Samarel AM, Im HJ (2003). Fibronectin fragment activation of proline-rich tyrosine kinase PYK2 mediates integrin signals regulating collagenase-3 expression by human chondrocytes through a protein kinase C-dependent pathway. *J Biol Chem* 278:24577-24585.

253. Lohmander LS, Dahlberg L, Eyre D, Lark M, Thonar EJ, Ryd L (1998). Longitudinal and cross-sectional variability in markers of joint metabolism in patients with knee pain and articular cartilage abnormalities. *Osteoarthritis Cartilage* 6:351-361.

254. Long P, Gassner R, Agarwal S (2001). Tumor necrosis factor alpha-dependent proinflammatory gene induction is inhibited by cyclic tensile strain in articular chondrocytes in vitro. *Arthritis Rheum* 44:2311-2319.

255. Long P, Liu F, Piesco NP, Kapur R, Agarwal S (2002). Signaling by mechanical strain involves transcriptional regulation of proinflammatory genes in human periodontal ligament cells in vitro. *Bone* 30:547-552.

256. Losman JA, Chen XP, Hilton D, Rothman P (1999). Cutting edge: SOCS-1 is a potent inhibitor of IL-4 signal transduction. *J Immunol* 162:3770-3774.

257. Lotz M, Blanco FJ, von Kempis J, Dudler J, Maier R, Villiger PM, Geng Y (1995). Cytokine regulation of chondrocyte functions. *J Rheumatol Suppl* 43:104-108.

258. Lucchinetti E, Bhargava MM, Torzilli PA (2004). The effect of mechanical load on integrin subunits alpha5 and beta1 in chondrocytes from mature and immature cartilage explants. *Cell Tissue Res* 315:385-391.

259. MacGillivray MK, Cruz TF, McCulloch CA (2000). The recruitment of the interleukin-1 (IL-1) receptor-associated kinase (IRAK) into focal adhesion complexes is required for IL-1beta -induced ERK activation. *J Biol Chem* 275:23509-23515.

260. MacKenna DA, Dolfi F, Vuori K, Ruoslahti E (1998). Extracellular signal-regulated kinase and c-Jun NH2-terminal kinase activation by mechanical stretch is integrin-dependent and matrix-specific in rat cardiac fibroblasts. *J Clin Invest* 101:301-310.

261. Maeda S, Dean DD, Sylvia VL, Boyan BD, Schwartz Z (2001). Metalloproteinase activity in growth plate chondrocyte cultures is regulated by 1,25-(OH)(2)D(3) and 24,25-(OH)(2)D(3) and mediated through protein kinase C. *Matrix Biol* 20:87-97.

262. Mahmood TA, de Jong R, Riesle J, Langer R, van Blitterswijk CA (2004). Adhesion-mediated signal transduction in human articular chondrocytes: the influence of biomaterial chemistry and tenascin-C. *Exp Cell Res* 301:179-188.

263. Malfait AM, Liu RQ, Ijiri K, Komiya S, Tortorella MD (2002). Inhibition of ADAM-TS4 and ADAM-TS5 prevents aggrecan degradation in osteoarthritic cartilage. *J Biol Chem* 277:22201-22208.

264. Mankin HJ, Dorfman H, Lippiello L, Zarins A (1971). Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. *J Bone Joint Surg Am* 53:523-537.

265. Marcus RE (1973). The effect of low oxygen concentration on growth, glycolysis, and sulfate incorporation by articular chondrocytes in monolayer culture. *Arthritis Rheum* 16:646-656.

270. Marks-Konczalik J, Chu SC, Moss J (1998). Cytokine-mediated transcriptional induction of the human inducible nitric oxide synthase gene requires both activator protein 1 and nuclear factor kappaB-binding sites. *J Biol Chem* 273:22201-22208.

271. Maroudas A, Venn M (1977). Chemical composition and swelling of normal and osteoarthrotic femoral head cartilage. II. Swelling. *Ann Rheum Dis* 36:399-406.

272. Maroudas AI (1976). Balance between swelling pressure and collagen tension in normal and degenerate cartilage. *Nature* 260:808-809.

273. Martel-Pelletier J, McCollum R, DiBattista J, Faure MP, Chin JA, Fournier S, Sarfati M, Pelletier JP (1992). The interleukin-1 receptor in normal and osteoarthritic human articular chondrocytes. Identification as the type I receptor and analysis of binding kinetics and biologic function. *Arthritis Rheum* 35:530-540.

274. Mashimo H, Goyal RK (1999). Lessons from genetically engineered animal models. IV. Nitric oxide synthase gene knockout mice. *Am J Physiol* 277:G745-G750.

275. Mathy-Hartert M, Deby-Dupont GP, Reginster JY, Ayache N, Pujol JP, Henrotin YE (2002). Regulation by reactive oxygen species of interleukin-1beta, nitric oxide and prostaglandin E(2) production by human chondrocytes. *Osteoarthritis Cartilage* 10:547-555.

276. Mathy-Hartert M, Martin G, Devel P, Deby-Dupont G, Pujol JP, Reginster JY, Henrotin Y (2003). Reactive oxygen species downregulate the expression of pro-inflammatory genes by human chondrocytes. *Inflamm Res* 52:111-118.

277. Matsubara M, Hayashi N, Jing T, Titani K (2003). Regulation of endothelial nitric oxide synthase by protein kinase C. *J Biochem* 133:773-781.

278. McCartney-Francis NL, Song X, Mizel DE, Wahl SM (2001). Selective inhibition of inducible nitric oxide synthase exacerbates erosive joint disease. *J Immunol* 166:2734-2740.

279. McCormick WF, Stewart JH (1983). Ossification patterns of costal cartilages as an indicator of sex. *Arch Pathol Lab Med* 107:206-210.

280. McInnes IB, Leung B, Wei XQ, Gemmell CC, Liew FY (1998). Septic arthritis following Staphylococcus aureus infection in mice lacking inducible nitric oxide synthase. *J Immunol* 160:308-315.

281. McMillan K, Adler M, Auld DS, Baldwin JJ, Blasko E, Browne LJ, Chelsky D, Davey D, Dolle RE, Eagen KA, Erickson S, Feldman RI, Glaser CB, Mallari C, Morrissey MM, Ohlmeyer MH, Pan G, Parkinson JF, Phillips GB, Polokoff MA, Sigal NH, Vergona R, Whitlow M, Young TA, Devlin JJ (2000). Allosteric inhibitors of inducible nitric oxide synthase dimerization discovered via combinatorial chemistry. *Proc Natl Acad Sci U S A* 97:1506-1511.

282. Meachim G, Denham D, Emery IH, Wilkinson PH (1974). Collagen alignments and artificial splits at the surface of human articular cartilage. *J Anat* 118:101-118.

283. Mehraban F, Kuo SY, Riera H, Chang C, Moskowitz RW (1994). Prostromelysin and procollagenase genes are differentially up-regulated in chondrocytes from the knees of rabbits with experimental osteoarthritis. *Arthritis Rheum* 37:1189-1197.

284. Meister A, Anderson ME (1983). Glutathione. *Annu Rev Biochem* 52:711-760.

285. Melchiorri C, Meliconi R, Frizziero L, Silvestri T, Pulsatelli L, Mazzetti I, Borzi RM, Uguccioni M, Facchini A (1998). Enhanced and coordinated in vivo expression of inflammatory cytokines and nitric oxide synthase by chondrocytes from patients with osteoarthritis. *Arthritis Rheum* 41:2165-2174.

286. Mendes AF, Caramona MM, Carvalho AP, Lopes MC (2002). Role of mitogen-activated protein kinases and tyrosine kinases on IL-1-Induced NF-kappaB activation and iNOS expression in bovine articular chondrocytes. *Nitric Oxide* 6:35-44.

287. Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D, Carver-Moore K, DuBois RN, Clark R, Aguet M, Schreiber RD (1996). Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* 84:431-442.

288. Michell BJ, Chen Z, Tiganis T, Stapleton D, Katsis F, Power DA, Sim AT, Kemp BE (2001). Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *J Biol Chem* 276:17625-17628.

289. Millward-Sadler SJ, Wright MO, Lee H, Nishida K, Caldwell H, Nuki G, Salter DM (1999). Integrin-regulated secretion of interleukin 4: A novel pathway of mechanotransduction in human articular chondrocytes. *J Cell Biol* 145:183-189.

290. Millward-Sadler SJ, Wright MO, Lee H, Caldwell H, Nuki G, Salter DM (2000a). Altered electrophysiological responses to mechanical stimulation and abnormal signalling through alpha5beta1 integrin in chondrocytes from osteoarthritic cartilage. *Osteoarthritis Cartilage* 8:272-278.

291. Millward-Sadler SJ, Wright MO, Davies LW, Nuki G, Salter DM (2000b). Mechanotransduction via integrins and interleukin-4 results in altered aggrecan and matrix metalloproteinase 3 gene expression in normal, but not osteoarthritic, human articular chondrocytes. *Arthritis Rheum* 43:2091-2099.

292. Millward-Sadler SJ, Mackenzie A, Wright MO, Lee HS, Elliot K, Gerrard L, Fiskerstrand CE, Salter DM, Quinn JP (2003). Tachykinin expression in cartilage

and function in human articular chondrocyte mechanotransduction. *Arthritis Rheum* 48:146-156.

293. Millward-Sadler SJ, Wright MO, Flatman PW, Salter DM (2004). ATP in the mechanotransduction pathway of normal human chondrocytes. *Biorheology* 41:567-575.

294. Min BH, Kim HJ, Lim H, Park CS, Park SR (2001). Effects of ageing and arthritic disease on nitric oxide production by human articular chondrocytes. *Exp Mol Med* 33:299-302.

295. Ming XF, Viswambharan H, Barandier C, Ruffieux J, Kaibuchi K, Rusconi S, Yang Z (2002). Rho GTPase/Rho kinase negatively regulates endothelial nitric oxide synthase phosphorylation through the inhibition of protein kinase B/Akt in human endothelial cells. *Mol Cell Biol* 22:8467-8477.

296. Mohtai M, Gupta MK, Donlon B, Ellison B, Cooke J, Gibbons G, Schurman DJ, Smith RL (1996). Expression of interleukin-6 in osteoarthritic chondrocytes and effects of fluid-induced shear on this expression in normal human chondrocytes in vitro. *J Orthop Res* 14:67-73.

297. Morales TI, Hascall VC (1989). Factors involved in the regulation of proteoglycan metabolism in articular cartilage. *Arthritis Rheum* 32:1197-1201.

298. Mort JS, Flannery CR, Makkerh J, Krupa JC, Lee ER (2003). Use of anti-neoepitope antibodies for the analysis of degradative events in cartilage and the molecular basis for neoepitope specificity. *Biochem Soc Symp*107-114.

299. Mullershausen F, Russwurm M, Thompson WJ, Liu L, Koesling D, Friebe A (2001). Rapid nitric oxide-induced desensitization of the cGMP response is caused by increased activity of phosphodiesterase type 5 paralleled by phosphorylation of the enzyme. *J Cell Biol* 155:271-278.

300. Murakami M, Narazaki M, Hibi M, Yawata H, Yasukawa K, Hamaguchi M, Taga T, Kishimoto T (1991). Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. *Proc Natl Acad Sci U S A* 88:11349-11353.

301. Murakami M, Hibi M, Nakagawa N, Nakagawa T, Yasukawa K, Yamanishi K, Taga T, Kishimoto T (1993). IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. *Science* 260:1808-1810.

302. Murakami S, Yoshimura N, Koide H, Watanabe J, Takedachi M, Terakura M, Yanagita M, Hashikawa T, Saho T, Shimabukuro Y, Okada H (2002). Activation of adenosine-receptor-enhanced iNOS mRNA expression by gingival epithelial cells. *J Dent Res* 81:236-240.

303. Murrell GA, Jang D, Williams RJ (1995). Nitric oxide activates metalloprotease enzymes in articular cartilage. *Biochem Biophys Res Commun* 206:15-21.

304. Musial A, Eissa NT (2001). Inducible nitric-oxide synthase is regulated by the proteasome degradation pathway. *J Biol Chem* 276:24268-24273.

305. Nanri K, Montecot C, Springhetti V, Seylaz J, Pinard E (1998). The selective inhibitor of neuronal nitric oxide synthase, 7-nitroindazole, reduces the delayed neuronal damage due to forebrain ischemia in rats. *Stroke* 29:1248-1253.

306. Nathan C, Xie QW (1994). Nitric oxide synthases: roles, tolls, and controls. *Cell* 78:915-918.

307. Nelson F, Dahlberg L, Laverty S, Reiner A, Pidoux I, Ionescu M, Fraser GL, Brooks E, Tanzer M, Rosenberg LC, Dieppe P, Robin PA (1998). Evidence for altered synthesis of type II collagen in patients with osteoarthritis. *J Clin Invest* 102:2115-2125.

308. Nemoto O, Yamada H, Kikuchi T, Shinmei M, Obata K, Sato H, Seiki M (1997). Suppression of matrix metalloproteinase-3 synthesis by interleukin-4 in human articular chondrocytes. *J Rheumatol* 24:1774-1779.

309. Notoya K, Jovanovic DV, Reboul P, Martel-Pelletier J, Mineau F, Pelletier JP (2000). The induction of cell death in human osteoarthritis chondrocytes by nitric oxide is related to the production of prostaglandin E2 via the induction of cyclooxygenase-2. *J Immunol* 165:3402-3410.

310. Novak A, Hsu SC, Leung-Hagesteijn C, Radeva G, Papkoff J, Montesano R, Roskelley C, Grosschedl R, Dedhar S (1998). Cell adhesion and the integrin-linked kinase regulate the LEF-1 and beta-catenin signaling pathways. *Proc Natl Acad Sci U S A* 95:4374-4379.

311. Nussler AK, Billiar TR, Liu ZZ, Morris SM, Jr. (1994). Coinduction of nitric oxide synthase and argininosuccinate synthetase in a murine macrophage cell line. Implications for regulation of nitric oxide production. *J Biol Chem* 269:1257-1261.

312. O'Hara BP, Urban JP, Maroudas A (1990). Influence of cyclic loading on the nutrition of articular cartilage. *Ann Rheum Dis* 49:536-539.

313. O'Neill LA (2002). Signal transduction pathways activated by the IL-1 receptor/toll-like receptor superfamily. *Curr Top Microbiol Immunol* 270:47-61.

314. Oh M, Fukuda K, Asada S, Yasuda Y, Tanaka S (1998). Concurrent generation of nitric oxide and superoxide inhibits proteoglycan synthesis in bovine articular chondrocytes: involvement of peroxynitrite. *J Rheumatol* 25:2169-2174.

315. Oh P, Schnitzer JE (2001). Segregation of heterotrimeric G proteins in cell surface microdomains. G(q) binds caveolin to concentrate in caveolae, whereas G(i) and G(s) target lipid rafts by default. *Mol Biol Cell* 12:685-698.

316. Ohmori Y, Smith MF, Jr., Hamilton TA (1996). IL-4-induced expression of the IL-1 receptor antagonist gene is mediated by STAT6. *J Immunol* 157:2058-2065.

317. Okada Y, Shinmei M, Tanaka O, Naka K, Kimura A, Nakanishi I, Bayliss MT, Iwata K, Nagase H (1992). Localization of matrix metalloproteinase 3 (stromelysin) in osteoarthritic cartilage and synovium. *Lab Invest* 66:680-690.

318. Ostergaard K, Petersen J, Andersen CB, Bendtzen K, Salter DM (1997a). Histologic/histochemical grading system for osteoarthritic articular cartilage: reproducibility and validity. *Arthritis Rheum* 40:1766-1771.

319. Ostergaard K, Salter DM, Andersen CB, Petersen J, Bendtzen K (1997b). CD44 expression is up-regulated in the deep zone of osteoarthritic cartilage from human femoral heads. *Histopathology* 31:451-459.

320. Ostergaard K, Salter DM, Petersen J, Bendtzen K, Hvolris J, Andersen CB (1998). Expression of alpha and beta subunits of the integrin superfamily in articular cartilage from macroscopically normal and osteoarthritic human femoral heads. *Ann Rheum Dis* 57:303-308.

321. Ouellet H, Ouellet Y, Richard C, Labarre M, Wittenberg B, Wittenberg J, Guertin M (2002). Truncated hemoglobin HbN protects Mycobacterium bovis from nitric oxide. *Proc Natl Acad Sci U S A* 99:5902-5907.

322. Ozaki K, Leonard WJ (2002). Cytokine and cytokine receptor pleiotropy and redundancy. *J Biol Chem* 277:29355-29358.

323. Palmer G, Guerne PA, Mezin F, Maret M, Guicheux J, Goldring MB, Gabay C (2002). Production of interleukin-1 receptor antagonist by human articular chondrocytes. *Arthritis Res* 4:226-231.

324. Palmer RM, Hickery MS, Charles IG, Moncada S, Bayliss MT (1993). Induction of nitric oxide synthase in human chondrocytes. *Biochem Biophys Res Commun* 193:398-405.

325. Palmoski MJ, Brandt KD (1981). Running inhibits the reversal of atrophic changes in canine knee cartilage after removal of a leg cast. *Arthritis Rheum* 24:1329-1337.

326. Pan J, Burgher KL, Szczepanik AM, Ringheim GE (1996). Tyrosine phosphorylation of inducible nitric oxide synthase: implications for potential post-translational regulation. *Biochem J* 314:889-894.

327. Pance A, Chantome A, Reveneau S, Bentrari F, Jeannin JF (2002). A repressor in the proximal human inducible nitric oxide synthase promoter modulates transcriptional activation. *FASEB J* 16:631-633.

328. Parkkinen JJ, Lammi MJ, Pelttari A, Helminen HJ, Tammi M, Virtanen I (1993). Altered Golgi apparatus in hydrostatically loaded articular cartilage chondrocytes. *Ann Rheum Dis* 52:192-198.

329. Parkkinen JJ, Lammi MJ, Inkinen R, Jortikka M, Tammi M, Virtanen I, Helminen HJ (1995). Influence of short-term hydrostatic pressure on organization of stress fibers in cultured chondrocytes. *J Orthop Res* 13:495-502.

330. Pelletier J, Jovanovic D, Fernandes JC, Manning P, Connor JR, Currie MG, Martel-Pelletier J (1999). Reduction in the structural changes of experimental osteoarthritis by a nitric oxide inhibitor. *Osteoarthritis Cartilage* 7:416-418.

331. Pelletier JP, Mineau F, Ranger P, Tardif G, Martel-Pelletier J (1996). The increased synthesis of inducible nitric oxide inhibits IL-1ra synthesis by human articular chondrocytes: possible role in osteoarthritic cartilage degradation. *Osteoarthritis Cartilage* 4:77-84.

332. Pelletier JP, Jovanovic DV, Lascau-Coman V, Fernandes JC, Manning PT, Connor JR, Currie MG, Martel-Pelletier J (2000). Selective inhibition of inducible nitric oxide synthase reduces progression of experimental osteoarthritis in vivo: possible link with the reduction in chondrocyte apoptosis and caspase 3 level. *Arthritis Rheum* 43:1290-1299.

333. Pollock JS, Forstermann U, Mitchell JA, Warner TD, Schmidt HH, Nakane M, Murad F (1991). Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc Natl Acad Sci U S A* 88:10480-10484.

334. Poole AR, Pidoux I, Reiner A, Tang LH, Choi H, Rosenberg L (1980). Localization of proteoglycan monomer and link protein in the matrix of bovine articular cartilage: An immunohistochemical study. *J Histochem Cytochem* 28:621-635.

335. Poole AR, Pidoux I, Reiner A, Rosenberg L (1982). An immunoelectron microscope study of the organization of proteoglycan monomer, link protein, and collagen in the matrix of articular cartilage. *J Cell Biol* 93:921-937.

336. Poole CA, Matsuoka A, Schofield JR (1991). Chondrons from articular cartilage. III. Morphologic changes in the cellular microenvironment of chondrons isolated from osteoarthritic cartilage. *Arthritis Rheum* 34:22-35.

337. Poole CA (1997). Articular cartilage chondrons: form, function and failure. *J Anat* 191 (Pt 1):1-13.

338. Presle N, Cipolletta C, Jouzeau JY, Abid A, Netter P, Terlain B (1999). Cartilage protection by nitric oxide synthase inhibitors after intraarticular injection of interleukin-1beta in rats. *Arthritis Rheum* 42:2094-2102.

339. Ragan PM, Badger AM, Cook M, Chin VI, Gowen M, Grodzinsky AJ, Lark MW (1999). Down-regulation of chondrocyte aggrecan and type-II collagen gene expression correlates with increases in static compression magnitude and duration. *J Orthop Res* 17:836-842.

340. Raman CS, Li H, Martasek P, Southan G, Masters BS, Poulos TL (2001). Crystal structure of nitric oxide synthase bound to nitro indazole reveals a novel inactivation mechanism. *Biochemistry* 40:13448-13455.

341. Rameau GA, Chiu LY, Ziff EB (2004). Bidirectional regulation of neuronal nitric oxide synthase phosphorylation at serine 847 by the N-methyl-D-aspartate receptor. *J Biol Chem* 279:14307-14314.

342. Ratovitski EA, Alam MR, Quick RA, McMillan A, Bao C, Kozlovsky C, Hand TA, Johnson RC, Mains RE, Eipper BA, Lowenstein CJ (1999a). Kalirin inhibition of inducible nitric-oxide synthase. *J Biol Chem* 274:993-999.

343. Ratovitski EA, Bao C, Quick RA, McMillan A, Kozlovsky C, Lowenstein CJ (1999b). An inducible nitric-oxide synthase (NOS)-associated protein inhibits NOS dimerization and activity. *J Biol Chem* 274:30250-30257.

344. Reginato AM, Olsen BR (2002). The role of structural genes in the pathogenesis of osteoarthritic disorders. *Arthritis Res* 4:337-345.

345. Relic B, Guicheux J, Mezin F, Lubberts E, Togninalli D, Garcia I, van den Berg WB, Guerne PA (2001). Il-4 and IL-13, but not IL-10, protect human synoviocytes from apoptosis. *J Immunol* 166:2775-2782.

346. Richards MK, Marletta MA (1994). Characterization of neuronal nitric oxide synthase and a C415H mutant, purified from a baculovirus overexpression system. *Biochemistry* 33:14723-14732.

347. Ridley SH, Sarsfield SJ, Lee JC, Bigg HF, Cawston TE, Taylor DJ, DeWitt DL, Saklatvala J (1997). Actions of IL-1 are selectively controlled by p38 mitogen-activated protein kinase: regulation of prostaglandin H synthase-2, metalloproteinases, and IL-6 at different levels. *J Immunol* 158:3165-3173.

348. Robbins JR, Thomas B, Tan L, Choy B, Arbiser JL, Berenbaum F, Goldring MB (2000). Immortalized human adult articular chondrocytes maintain cartilage-specific phenotype and responses to interleukin-1beta. *Arthritis Rheum* 43:2189-2201.

349. Roberts SR, Knight MM, Lee DA, Bader DL (2001). Mechanical compression influences intracellular Ca2+ signaling in chondrocytes seeded in agarose constructs. *J Appl Physiol* 90:1385-1391.

350. Rodriguez-Pascual F, Hausding M, Ihrig-Biedert I, Furneaux H, Levy AP, Forstermann U, Kleinert H (2000). Complex contribution of the 3'-untranslated region to the expressional regulation of the human inducible nitric-oxide synthase gene. Involvement of the RNA-binding protein HuR. *J Biol Chem* 275:26040-26049.

351. Roman LJ, Martasek P, Miller RT, Harris DE, de La Garza MA, Shea TM, Kim JJ, Masters BS (2000a). The C termini of constitutive nitric-oxide synthases control electron flow through the flavin and heme domains and affect modulation by calmodulin. *J Biol Chem* 275:29225-29232.

352. Roman LJ, Miller RT, de La Garza MA, Kim JJ, Siler Masters BS (2000b). The C terminus of mouse macrophage inducible nitric-oxide synthase attenuates electron flow through the flavin domain. *J Biol Chem* 275:21914-21919.

353. Roman LJ, Martasek P, Masters BS (2002). Intrinsic and extrinsic modulation of nitric oxide synthase activity. *Chem Rev* 102:1179-1190.

354. Rosado E, Schwartz Z, Sylvia VL, Dean DD, Boyan BD (2002). Transforming growth factor-beta1 regulation of growth zone chondrocytes is mediated by multiple interacting pathways. *Biochim Biophys Acta* 1590:1-15.

355. Roughley PJ (2001). Articular cartilage and changes in arthritis: noncollagenous proteins and proteoglycans in the extracellular matrix of cartilage. *Arthritis Res* 3:342-347.

356. Ruwhof C, van der Laarse A (2000). Mechanical stress-induced cardiac hypertrophy: mechanisms and signal transduction pathways. *Cardiovasc Res* 47:23-37.

357. Sakai A, Hirano T, Okazaki R, Okimoto N, Tanaka K, Nakamura T (1999). Large-dose ascorbic acid administration suppresses the development of arthritis in adjuvant-infected rats. *Arch Orthop Trauma Surg* 119:121-126.

358. Salerno JC, Harris DE, Irizarry K, Patel B, Morales AJ, Smith SM, Martasek P, Roman LJ, Masters BS, Jones CL, Weissman BA, Lane P, Liu Q, Gross SS (1997). An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase. *J Biol Chem* 272:29769-29777.

359. Salter DM, Hughes DE, Simpson R, Gardner DL (1992). Integrin expression by human articular chondrocytes. *Br J Rheumatol* 31:231-234.

360. Salter DM (1993). Tenascin is increased in cartilage and synovium from arthritic knees. *Br J Rheumatol* 32:780-786.

361. Salter DM, Robb JE, Wright MO (1997). Electrophysiological responses of human bone cells to mechanical stimulation: evidence for specific integrin function in mechanotransduction. *J Bone Miner Res* 12:1133-1141.

362. Salter DM, Millward-Sadler SJ, Nuki G, Wright MO (2002). Differential responses of chondrocytes from normal and osteoarthritic human articular cartilage to mechanical stimulation. *Biorheology* 39:97-108.

363. Salter DM, Wright MO, Millward-Sadler SJ (2004). NMDA receptor expression and roles in human articular chondrocyte mechanotransduction. *Biorheology* 41:273-281.

364. Salzman A, Denenberg AG, Ueta I, O'Connor M, Linn SC, Szabo C (1996). Induction and activity of nitric oxide synthase in cultured human intestinal epithelial monolayers. *Am J Physiol* 270:G565-G573.

365. Sanchez C, Mateus MM, Defresne MP, Crielaard JM, Reginster JY, Henrotin YE (2002). Metabolism of human articular chondrocytes cultured in alginate beads. Longterm effects of interleukin 1beta and nonsteroidal antiinflammatory drugs. *J Rheumatol* 29:772-782.

366. Sandell LJ, Aigner T (2001). Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res* 3:107-113.

367. Sasaki K, Hattori T, Fujisawa T, Takahashi K, Inoue H, Takigawa M (1998). Nitric oxide mediates interleukin-1-induced gene expression of matrix metalloproteinases and basic fibroblast growth factor in cultured rabbit articular chondrocytes. *J Biochem (Tokyo)* 123:431-439.

368. Sauerland K, Raiss RX, Steinmeyer J (2003). Proteoglycan metabolism and viability of articular cartilage explants as modulated by the frequency of intermittent loading. *Osteoarthritis Cartilage* 11:343-350.

369. Saura M, Zaragoza C, Bao C, McMillan A, Lowenstein CJ (1999). Interaction of interferon regulatory factor-1 and nuclear factor kappaB during activation of inducible nitric oxide synthase transcription. *J Mol Biol* 289:459-471.

370. Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, Parsons JT (1994). Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol* 14:1680-1688.

371. Schlaepfer DD, Hanks SK, Hunter T, van der GP (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* 372:786-791.

372. Schlaepfer DD, Broome MA, Hunter T (1997). Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. *Mol Cell Biol* 17:1702-1713.

373. Schreck R, Rieber P, Baeuerle PA (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J* 10:2247-2258.

374. Schuringa JJ, Jonk LJ, Dokter WH, Vellenga E, Kruijer W (2000). Interleukin-6-induced STAT3 transactivation and Ser727 phosphorylation involves Vav, Rac-1 and the kinase SEK-1/MKK-4 as signal transduction components. *Biochem J* 347 Pt 1:89-96.

375. Schwachtgen JL, Houston P, Campbell C, Sukhatme V, Braddock M (1998). Fluid shear stress activation of egr-1 transcription in cultured human endothelial and epithelial cells is mediated via the extracellular signal-related kinase 1/2 mitogen-activated protein kinase pathway. *J Clin Invest* 101:2540-2549.

376. Schwartz MA (2001). Integrin signaling revisited. *Trends Cell Biol* 11:466-470.

377. Seguin CA, Bernier SM (2003). TNFalpha suppresses link protein and type II collagen expression in chondrocytes: Role of MEK1/2 and NF-kappaB signaling pathways. *J Cell Physiol* 197:356-369.

378. Shakibaei M, Mobasheri A (2003). Beta1-integrins co-localize with Na, K-ATPase, epithelial sodium channels (ENaC) and voltage activated calcium channels (VACC) in mechanoreceptor complexes of mouse limb-bud chondrocytes. *Histol Histopathol* 18:343-351.

379. Sharma L (2001). Local factors in osteoarthritis. *Curr Opin Rheumatol* 13:441-446.

380. Shi CS, Kehrl JH (1997). Activation of stress-activated protein kinase/c-Jun N-terminal kinase, but not NF-kappaB, by the tumor necrosis factor (TNF) receptor 1 through a TNF receptor-associated factor 2- and germinal center kinase related-dependent pathway. *J Biol Chem* 272:32102-32107.

381. Shuai K, Schindler C, Prezioso VR, Darnell JE, Jr. (1992). Activation of transcription by IFN-gamma: tyrosine phosphorylation of a 91-kD DNA binding protein. *Science* 258:1808-1812.

382. Shyy JY, Chien S (1997). Role of integrins in cellular responses to mechanical stress and adhesion. *Curr Opin Cell Biol* 9:707-713.

383. Siddhanta U, Wu C, Abu-Soud HM, Zhang J, Ghosh DK, Stuehr DJ (1996). Heme iron reduction and catalysis by a nitric oxide synthase heterodimer containing one reductase and two oxygenase domains. *J Biol Chem* 271:7309-7312.

384. Silacci P, Dayer JM, Desgeorges A, Peter R, Manueddu C, Guerne PA (1998). Interleukin (IL)-6 and its soluble receptor induce TIMP-1 expression in

synoviocytes and chondrocytes, and block IL-1-induced collagenolytic activity. *J Biol Chem* 273:13625-13629.

385. Siow YL, Kalmar GB, Sanghera JS, Tai G, Oh SS, Pelech SL (1997). Identification of two essential phosphorylated threonine residues in the catalytic domain of Mekk1. Indirect activation by Pak3 and protein kinase C. *J Biol Chem* 272:7586-7594.

386. Sironen RK, Karjalainen HM, Torronen K, Elo MA, Kaarniranta K, Takigawa M, Helminen HJ, Lammi MJ (2002). High pressure effects on cellular expression profile and mRNA stability. A cDNA array analysis. *Biorheology* 39:111-117.

387. Slack J, McMahan CJ, Waugh S, Schooley K, Spriggs MK, Sims JE, Dower SK (1993). Independent binding of interleukin-1 alpha and interleukin-1 beta to type I and type II interleukin-1 receptors. *J Biol Chem* 268:2513-2524.

388. Slack JL, Schooley K, Bonnert TP, Mitcham JL, Qwarnstrom EE, Sims JE, Dower SK (2000). Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways. *J Biol Chem* 275:4670-4678.

389. Smith RL, Donlon BS, Gupta MK, Mohtai M, Das P, Carter DR, Cooke J, Gibbons G, Hutchinson N, Schurman DJ (1995). Effects of fluid-induced shear on articular chondrocyte morphology and metabolism *in vitro*. *J Orthop Res* 13:824-831.

390. Smith RL, Rusk SF, Ellison BE, Wessells P, Tsuchiya K, Carter DR, Caler WE, Sandell LJ, Schurman DJ (1996). In vitro stimulation of articular chondrocyte mRNA and extracellular matrix synthesis by hydrostatic pressure. *J Orthop Res* 14:53-60.

391. Smith RL, Lin J, Trindade MC, Shida J, Kajiyama G, Vu T, Hoffman AR, van der Meulen MC, Goodman SB, Schurman DJ, Carter DR (2000). Time-dependent effects of intermittent hydrostatic pressure on articular chondrocyte type II collagen and aggrecan mRNA expression. *J Rehabil Res Dev* 37:153-161.

392. Sohen S, Ooe H, Hashima M, Nonaka T, Fukuda K, Hamanishi C (2001). Activation of histamine H1 receptor results in enhanced proteoglycan synthesis by human articular chondrocyte: involvement of protein kinase C and intracellular Ca(2+). *Pathophysiology* 8:93-98.

393. Songu-Mize E, Liu X, Hymel LJ (1998). Effect of mechanical strain on expression of Na+,K+-ATPase alpha subunits in rat aortic smooth muscle cells. *Am J Med Sci* 316:196-199.

394. Spreng D, Sigrist N, Schweighauser A, Busato A, Schawalder P (2001). Endogenous nitric oxide production in canine osteoarthritis: Detection in urine, serum, and synovial fluid specimens. *Vet Surg* 30:191-199.

395. Stadler J, Stefanovic-Racic M, Billiar TR, Curran RD, McIntyre LA, Georgescu HI, Simmons RL, Evans CH (1991). Articular chondrocytes synthesize nitric oxide in response to cytokines and lipopolysaccharide. *J Immunol* 147:3915-3920.

396. Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, Quelle FW, Silvennoinen O, Barbieri G, Pellegrini S (1994). Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. *Science* 263:92-95.

397. Stamler JS (1994). Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell* 78:931-936.

398. Stanton LA, Underhill TM, Beier F (2003). MAP kinases in chondrocyte differentiation. *Dev Biol* 263:165-175.

399. Stefanovic-Racic M, Stadler J, Georgescu HI, Evans CH (1994). Nitric oxide and energy production in articular chondrocytes. *J Cell Physiol* 159:274-280.

400. Stefanovic-Racic M, Meyers K, Meschter C, Coffey JW, Hoffman RA, Evans CH (1995). Comparison of the nitric oxide synthase inhibitors methylarginine and aminoguanidine as prophylactic and therapeutic agents in rat adjuvant arthritis. *J Rheumatol* 22:1922-1928.

401. Stefanovic-Racic M, Morales TI, Taskiran D, McIntyre LA, Evans CH (1996). The role of nitric oxide in proteoglycan turnover by bovine articular cartilage organ cultures. *J Immunol* 156:1213-1220.

402. Stefanovic-Racic M, Mollers MO, Miller LA, Evans CH (1997). Nitric oxide and proteoglycan turnover in rabbit articular cartilage. *J Orthop Res* 15:442-449.

403. Steffan NM, Bren GD, Frantz B, Tocci MJ, O'Neill EA, Paya CV (1995). Regulation of IkB alpha phosphorylation by PKC- and Ca(2+)-dependent signal transduction pathways. *J Immunol* 155:4685-4691.

404. Studer R, Jaffurs D, Stefanovic-Racic M, Robbins PD, Evans CH (1999). Nitric oxide in osteoarthritis. *Osteoarthritis Cartilage* 7:377-379.

405. Studer RK, Levicoff E, Georgescu H, Miller L, Jaffurs D, Evans CH (2000). Nitric oxide inhibits chondrocyte response to IGF-I: inhibition of IGF-IRbeta tyrosine phosphorylation. *Am J Physiol Cell Physiol* 279:C961-C969.

406. Studer RK (2004). Nitric oxide decreases IGF-1 receptor function in vitro; glutathione depletion enhances this effect in vivo. *Osteoarthritis Cartilage* 12:863-869.

407. Sugden PH (2003). Ras, Akt, and mechanotransduction in the cardiac myocyte. *Circ Res* 93:1179-1192.

408. Sundberg C, Rubin K (1996). Stimulation of beta1 integrins on fibroblasts induces PDGF independent tyrosine phosphorylation of PDGF beta-receptors. *J Cell Biol* 132:741-752.

409. Swoboda B, Pullig O, Kirsch T, Kladny B, Steinhauser B, Weseloh G (1998). Increased content of type-VI collagen epitopes in human osteoarthritic cartilage: quantitation by inhibition ELISA. *J Orthop Res* 16:96-99.

410. Tamura T, Nakanishi T, Kimura Y, Hattori T, Sasaki K, Norimatsu H, Takahashi K, Takigawa M (1996). Nitric oxide mediates interleukin-1-induced matrix degradation and basic fibroblast growth factor release in cultured rabbit articular chondrocytes: a possible mechanism of pathological neovascularization in arthritis. *Endocrinology* 137:3729-3737.

411. Tang JL, Zembowicz A, Xu XM, Wu KK (1995). Role of Sp1 in transcriptional activation of human nitric oxide synthase type III gene. *Biochem Biophys Res Commun* 213:673-680.

412. Taskiran D, Stefanovic-Racic M, Georgescu H, Evans C (1994). Nitric oxide mediates suppression of cartilage proteoglycan synthesis by interleukin-1. *Biochem Biophys Res Commun* 200:142-148.

413. Taylor BS, de Vera ME, Ganster RW, Wang Q, Shapiro RA, Morris SM, Jr., Billiar TR, Geller DA (1998). Multiple NF-kappaB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J Biol Chem* 273:15148-15156.

414. Thompson RC, Jr., Oegema TR, Jr. (1979). Metabolic activity of articular cartilage in osteoarthritis. An in vitro study. *J Bone Joint Surg Am* 61:407-416.

415. Thum T, Tsikas D, Frolich JC, Borlak J (2003). Growth hormone induces eNOS expression and nitric oxide release in a cultured human endothelial cell line. *FEBS Lett* 555:567-571.

416. Tiku ML, Shah R, Allison GT (2000). Evidence linking chondrocyte lipid peroxidation to cartilage matrix protein degradation. Possible role in cartilage aging and the pathogenesis of osteoarthritis. *J Biol Chem* 275:20069-20076.

417. Tinker AC, Beaton HG, Boughton-Smith N, Cook TR, Cooper SL, Fraser-Rae L, Hallam K, Hamley P, McInally T, Nicholls DJ, Pimm AD, Wallace AV (2003). 1,2-Dihydro-4-quinazolinamines: potent, highly selective inhibitors of inducible nitric oxide synthase which show antiinflammatory activity in vivo. *J Med Chem* 46:913-916.

418. Tomita M, Sato EF, Nishikawa M, Yamano Y, Inoue M (2001). Nitric oxide regulates mitochondrial respiration and functions of articular chondrocytes. *Arthritis Rheum* 44:96-104.

419. Tortorella MD, Burn TC, Pratta MA, Abbaszade I, Hollis JM, Liu R, Rosenfeld SA, Copeland RA, Decicco CP, Wynn R, Rockwell A, Yang F, Duke JL, Solomon K, George H, Bruckner R, Nagase H, Itoh Y, Ellis DM, Ross H, Wiswall BH, Murphy K, Hillman MC, Jr., Hollis GF, Arner EC, . (1999). Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins. *Science* 284:1664-1666.

420. Towle CA, Hung HH, Bonassar LJ, Treadwell BV, Mangham DC (1997). Detection of interleukin-1 in the cartilage of patients with osteoarthritis: a possible autocrine/paracrine role in pathogenesis. *Osteoarthritis Cartilage* 5:293-300.

421. Toyoda T, Seedhom BB, Yao JQ, Kirkham J, Brookes S, Bonass WA (2003). Hydrostatic pressure modulates proteoglycan metabolism in chondrocytes seeded in agarose. *Arthritis Rheum* 48:2865-2872.

422. Tuna M, Polat S, Erman T, Ildan F, Gocer AI, Tuna N, Tamer L, Kaya M, Cetinalp E (2001). Effect of anti-rat interleukin-6 antibody after spinal cord injury in the rat: inducible nitric oxide synthase expression, sodium- and potassium-activated, magnesium-dependent adenosine-5'-triphosphatase and superoxide dismutase activation, and ultrastructural changes. *J Neurosurg Spine* 95:64-73.

423. Urban JP, Hall AC, Gehl KA (1993). Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes. *J Cell Physiol* 154:262-270.

424. Urban JP (1994). The chondrocyte: a cell under pressure. *Br J Rheumatol* 33:901-908.

425. Valhmu WB, Stazzone EJ, Bachrach NM, Saed-Nejad F, Fischer SG, Mow VC, Ratcliffe A (1998). Load-controlled compression of articular cartilage induces a transient stimulation of aggrecan gene expression. *Arch Biochem Biophys* 353:29-36.

426. van't Hof RJ, Hocking L, Wright PK, Ralston SH (2000). Nitric oxide is a mediator of apoptosis in the rheumatoid joint. *Rheumatology (Oxford)* 39:1004-1008.

427. van't Hof RJ, Armour KJ, Smith LM, Armour KE, Wei XQ, Liew FY, Ralston SH (2000). Requirement of the inducible nitric oxide synthase pathway for IL-1-induced osteoclastic bone resorption. *Proc Natl Acad Sci U S A* 97:7993-7998.

428. van de Loo FA, Kuiper S, van Enckevort FH, Arntz OJ, van den Berg WB (1997b). Interleukin-6 reduces cartilage destruction during experimental arthritis. A study in interleukin-6-deficient mice. *Am J Pathol* 151:177-191.

429. van de Loo FA, Arntz OJ, van den Berg WB (1997a). Effect of interleukin 1 and leukaemia inhibitory factor on chondrocyte metabolism in articular cartilage from

normal and interleukin-6-deficient mice: role of nitric oxide and IL-6 in the suppression of proteoglycan synthesis. *Cytokine* 9:453-462.

430. van den Berg WB, van de LF, Joosten LA, Arntz OJ (1999). Animal models of arthritis in NOS2-deficient mice. *Osteoarthritis Cartilage* 7:413-415.

431. van Lent PL, Holthuysen AE, Sloetjes A, Lubberts E, van den Berg WB (2002). Local overexpression of adeno-viral IL-4 protects cartilage from metallo proteinase-induced destruction during immune complex-mediated arthritis by preventing activation of pro-MMPs. *Osteoarthritis Cartilage* 10:234-243.

432. Veihelmann A, Landes J, Hofbauer A, Dorger M, Refior HJ, Messmer K, Krombach F (2001). Exacerbation of antigen-induced arthritis in inducible nitric oxide synthase-deficient mice. *Arthritis Rheum* 44:1420-1427.

433. Veihelmann A, Hofbauer A, Krombach F, Dorger M, Maier M, Refior HJ, Messmer K (2002). Differential function of nitric oxide in murine antigen-induced arthritis. *Rheumatology (Oxford)* 41:509-517.

434. Vincent TL, Hermansson MA, Hansen UN, Amis AA, Saklatvala J (2004). Basic fibroblast growth factor mediates transduction of mechanical signals when articular cartilage is loaded. *Arthritis Rheum* 50:526-533.

435. von der MK, Kirsch T, Nerlich A, Kuss A, Weseloh G, Gluckert K, Stoss H (1992). Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy. *Arthritis Rheum* 35:806-811.

436. Vuolteenaho K, Moilanen T, Hamalainen M, Moilanen E (2003). Regulation of nitric oxide production in osteoarthritic and rheumatoid cartilage. Role of endogenous IL-1 inhibitors. *Scand J Rheumatol* 32:19-24.

437. Vuori K, Ruoslahti E (1994). Association of insulin receptor substrate-1 with integrins. *Science* 266:1576-1578.

438. Vuori K, Hirai H, Aizawa S, Ruoslahti E (1996). Introduction of p130cas signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. *Mol Cell Biol* 16:2606-2613.

439. Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Hong J, Kandel RA (2003). Effect of biomechanical conditioning on cartilaginous tissue formation in vitro. *J Bone Joint Surg Am* 85-A Suppl 2:101-105.

440. Wang HG, Rapp UR, Reed JC (1996). Bcl-2 targets the protein kinase Raf-1 to mitochondria. *Cell* 87:629-638.

441. Wang L, Zhou P, Craig RW, Lu L (1999). Protection from cell death by mcl-1 is mediated by membrane hyperpolarization induced by K(+) channel activation. *J Membr Biol* 172:113-120.

442. Wang N and Ingber DE (1995). Probing transmembrane mechanical coupling and cytomechanics using magnetic twisting cytometry. *Biochem Cell Biol* 73:327-335.

443. Wang Z, Brecher P (1999). Salicylate inhibition of extracellular signal-regulated kinases and inducible nitric oxide synthase. *Hypertension* 34:1259-1264.

444. Watkins SC, Macaulay W, Turner D, Kang R, Rubash HE, Evans CH (1997). Identification of inducible nitric oxide synthase in human macrophages surrounding loosened hip prostheses. *Am J Pathol* 150:1199-1206.

445. Watling D, Guschin D, Muller M, Silvennoinen O, Witthuhn BA, Quelle FW, Rogers NC, Schindler C, Stark GR, Ihle JN, . (1993). Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-gamma signal transduction pathway. *Nature* 366:166-170.

446. Webb GR, Westacott CI, Elson CJ (1997). Chondrocyte tumor necrosis factor receptors and focal loss of cartilage in osteoarthritis. *Osteoarthritis Cartilage* 5:427-437.

447. Wei XQ, Charles IG, Smith A, Ure J, Feng GJ, Huang FP, Xu D, Muller W, Moncada S, Liew FY (1995). Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* 375:408-411.

448. Werb Z, Tremble PM, Behrendtsen O, Crowley E, Damsky CH (1989). Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *J Cell Biol* 109:877-889.

449. Westacott CI, Whicher JT, Barnes IC, Thompson D, Swan AJ, Dieppe PA (1990). Synovial fluid concentration of five different cytokines in rheumatic diseases. *Ann Rheum Dis* 49:676-681.

450. Westacott CI, Barakat AF, Wood L, Perry MJ, Neison P, Bisbinas I, Armstrong L, Millar AB, Elson CJ (2000). Tumor necrosis factor alpha can contribute to focal loss of cartilage in osteoarthritis. *Osteoarthritis Cartilage* 8:213-221.

451. Wink DA, Cook JA, Pacelli R, DeGraff W, Gamson J, Liebmann J, Krishna MC, Mitchell JB (1996a). The effect of various nitric oxide-donor agents on hydrogen peroxide-mediated toxicity: a direct correlation between nitric oxide formation and protection. *Arch Biochem Biophys* 331:241-248.

452. Wink DA, Hanbauer I, Grisham MB, Laval F, Nims RW, Laval J, Cook J, Pacelli R, Liebmann J, Krishna M, Ford PC, Mitchell JB (1996b). Chemical biology of nitric oxide: regulation and protective and toxic mechanisms. *Curr Top Cell Regul* 34:159-187.

453. Wiseman M, Henson F, Lee DA, Bader DL (2003). Dynamic compressive strain inhibits nitric oxide synthesis by equine chondrocytes isolated from different areas of the cartilage surface. *Equine Vet J* 35:451-456.

454. Wolff J, Wong C, Cheng H, Poyet P, Butel JS, Rosen JM (1992). Differential effects of the simian virus 40 early genes on mammary epithelial cell growth, morphology, and gene expression. *Exp Cell Res* 202:67-76.

455. Wong HL, Costa GL, Lotze MT, Wahl SM (1993). Interleukin (IL) 4 differentially regulates monocyte IL-1 family gene expression and synthesis in vitro and in vivo. *J Exp Med* 177:775-781.

456. Wong M, Siegrist M, Cao X (1999). Cyclic compression of articular cartilage explants is associated with progressive consolidation and altered expression pattern of extracellular matrix proteins. *Matrix Biol* 18:391-399.

457. Wright M, Jobanputra P, Bavington C, Salter DM, Nuki G (1996). Effects of intermittent pressure-induced strain on the electrophysiology of cultured human chondrocytes: evidence for the presence of stretch-activated membrane ion channels. *Clin Sci (Lond)* 90:61-71.

458. Wright MO, Stockwell RA, Nuki G (1992). Response of plasma membrane to applied hydrostatic pressure in chondrocytes and fibroblasts. *Connect Tissue Res* 28:49-70.

459. Wright MO, Nishida K, Bavington C, Godolphin JL, Dunne E, Walmsley S, Jobanputra P, Nuki G, Salter DM (1997). Hyperpolarisation of cultured human chondrocytes following cyclical pressure-induced strain: evidence of a role for alpha 5 beta 1 integrin as a chondrocyte mechanoreceptor. *J Orthop Res* 15:742-747.

460. Wu Q, Zhang Y, Chen Q (2001). Indian hedgehog is an essential component of mechanotransduction complex to stimulate chondrocyte proliferation. *J Biol Chem* 276:35290-35296.

461. Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, Nathan C (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* 256:225-228.

462. Xu JW, Zaporojan V, Peretti GM, Roses RE, Morse KB, Roy AK, Mesa JM, Randolph MA, Bonassar LJ, Yaremchuk MJ (2004). Injectable tissue-engineered cartilage with different chondrocyte sources. *Plast Reconstr Surg* 113:1361-1371.

463. Xu Z, Buckley MJ, Evans CH, Agarwal S (2000). Cyclic tensile strain acts as an antagonist of IL-1 beta actions in chondrocytes. *J Immunol* 165:453-460.

464. Yamamoto K, Dang QN, Kelly RA, Lee RT (1998). Mechanical strain suppresses inducible nitric-oxide synthase in cardiac myocytes. *J Biol Chem* 273:11862-11866.

465. Yin MJ, Christerson LB, Yamamoto Y, Kwak YT, Xu S, Mercurio F, Barbosa M, Cobb MH, Gaynor RB (1998). HTLV-I Tax protein binds to MEKK1 to stimulate IkappaB kinase activity and NF-kappaB activation. *Cell* 93:875-884.

466. Yokota H, Goldring MB, Sun HB (2003). CITED2-mediated regulation of MMP-1 and MMP-13 in human chondrocytes under flow shear. *J Biol Chem* 278:47275-47280.

467. Yoon YM, Kim SJ, Oh CD, Ju JW, Song WK, Yoo YJ, Huh TL, Chun JS (2002). Maintenance of differentiated phenotype of articular chondrocytes by protein kinase C and extracellular signal-regulated protein kinase. *J Biol Chem* 277:8412-8420.

468. Young RJ, Beams RM, Carter K, Clark HA, Coe DM, Chambers CL, Davies PI, Dawson J, Drysdale MJ, Franzman KW, French C, Hodgson ST, Hodson HF, Kleanthous S, Rider P, Sanders D, Sawyer DA, Scott KJ, Shearer BG, Stocker R, Smith S, Tackley MC, Knowles RG (2000). Inhibition of inducible nitric oxide synthase by acetamidine derivatives of hetero-substituted lysine and homolysine. *Bioorg Med Chem Lett* 10:597-600.

469. Yu Z, Zhang W, Kone BC (2002a). Histone deacetylases augment cytokine induction of the iNOS gene. *J Am Soc Nephrol* 13:2009-2017.

470. Yu Z, Zhang W, Kone BC (2002b). Signal transducers and activators of transcription 3 (STAT3) inhibits transcription of the inducible nitric oxide synthase gene by interacting with nuclear factor kappaB. *Biochem J* 367:97-105.

471. Yuasa T, Ohno S, Kehrl JH, Kyriakis JM (1998). Tumor necrosis factor signaling to stress-activated protein kinase (SAPK)/Jun NH2-terminal kinase (JNK) and p38. Germinal center kinase couples TRAF2 to mitogen-activated protein kinase/ERK kinase kinase 1 and SAPK while receptor interacting protein associates with a mitogen-activated protein kinase kinase kinase upstream of MKK6 and p38. *J Biol Chem* 273:22681-22692.

472. Zaleskas JM, Kinner B, Freyman TM, Yannas IV, Gibson LJ, Spector M (2004). Contractile forces generated by articular chondrocytes in collagen-glycosaminoglycan matrices. *Biomaterials* 25:1299-1308.

473. Zhang W, Kuncewicz T, Yu ZY, Zou L, Xu X, Kone BC (2003). Protein-protein interactions involving inducible nitric oxide synthase. *Acta Physiol Scand* 179:137-142.

474. Zhang X, Wrzeszczynska MH, Horvath CM, Darnell JE, Jr. (1999). Interacting regions in Stat3 and c-Jun that participate in cooperative transcriptional activation. *Mol Cell Biol* 19:7138-7146.

475. Zhang Y, Dawson VL, Dawson TM (2000). Oxidative stress and genetics in the pathogenesis of Parkinson's disease. *Neurobiol Dis* 7:240-250.

476. Zhang Y, Rosenberg PA (2002). The essential nutrient pyrroloquinoline quinone may act as a neuroprotectant by suppressing peroxynitrite formation. *Eur J Neurosci* 16:1015-1024.

477. Nicola NA. Guidebook to Cytokines and their receptors. Sambrook and Tooze Publications. Oxford University Press Inc., New York (1997).